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........................................ (Signed)

CHENGYUAN WANG

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PHARMACEUTICAL STUDIES ON LIGUSTILIDE

Submitted by

CHENGYUAN WANG

For the degree of Doctor of Philosophy

At the Hong Kong Polytechnic University in June 2007

ABSTRACT

Ligustilide (LIG) is the main effective component in Angelica sinensis (当归) and Ligusticum chuanxiong (川芎). Previous studies had shown that LIG had significant pharmacological effects on cerebral blood vessels, the general circulatory system and immune system. LIG in the medicinal materials has two configurations, Z-ligustilide (Z-LIG) and E-ligustilide (E-LIG). Z-LIG is the main configuration. However, since LIG has a poor stability, it is very difficult to prepare and preserve it. Therefore, the study of LIG has been limited and so far is not comprehensive and systematic. In this thesis, we seek solutions for these problems. In the first series of experiments we systematically examined the content of LIG in Angelica sinensis and Ligusticum chuanxiong, and found that the former is the better source of LIG. We then systematically extract and isolate LIG in Angelica sinensis volatile oil (ASVO), and discovered a new isolation method of LIG that is suitable for industrial production. A patent has been granted for this new isolation
method, which produces high purity LIG that could be used as the standard and also the raw material for a new medicine in first category. We also tried to increase the yield of synthesis of LIG but the yield was too low for larger scale industrial production. In the second series of experiments we characterized the physical and chemical properties as well as the stability of LIG systematically based on the high purity LIG. We also searched for specific stabilizer or any factor that could increase the stability of LIG. Based on these experiments we developed the highly stable LIG which could be used as the standard in the industrial production. Furthermore, we developed cyclodextrin (CD) inclusion complex-based and micro-emulsion (ME) of LIG preparations, with enhanced stability of LIG in both preparations, laying the foundation for the development of different LIG preparations. Finally we studied the acute toxicity of LIG, which was found to be low and complied with the safety requirement of new medicine. Therefore, LIG has good potential to be developed into a new first category medicine in the future. This thesis consists of 11 chapters, beginning with a general introduction, and then followed by the methodology section. There are then eight chapters on results, and at the end, there is a general discussion.
LIST OF PUBLICATIONS

SCI Papers


Pharmacol. 69(3): 968-974.


**National Journal Papers**


Conference Abstracts or Papers


**Patents**


2. Qian ZM, **Wang CY**, Du JR. Clathrate compound of ligustilide, cyclodextrin or its derivative, its formulation and pharmaceutical preparation. Patent application number: CN 200510021303.2. Patent application date: July 15, 2005


4. Qian ZM, Du JR, **Wang CY**. Application of ligustilide to prepare the medicine
for preventing and treating dysmenorrhea. Patent application number: CN 200510021300.9. Patent application date: July 15, 2005

5. Qian ZM, Du JR, Wang CY. Application of ligustilide to prepare the medicine for preventing and treating ischemic disease. Patent application number: CN 200510021301.3. Patent application date: July 15, 2005
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ASVO</td>
<td>Angelica sinensis volatile oil</td>
</tr>
<tr>
<td>BuLi</td>
<td>Butyl Lithium</td>
</tr>
<tr>
<td>CCD</td>
<td>Central composite design</td>
</tr>
<tr>
<td>CD</td>
<td>Cyclodextrin</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>DAD</td>
<td>Diode array detector</td>
</tr>
<tr>
<td>d</td>
<td>Day(s)</td>
</tr>
<tr>
<td>DCC</td>
<td>Decompression column chromatography</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>ED</td>
<td>Effective dose</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-related kinases</td>
</tr>
<tr>
<td>E-LIG</td>
<td>E-ligustilide</td>
</tr>
<tr>
<td>FCI</td>
<td>Forebrain cerebral ischemia</td>
</tr>
<tr>
<td>FID</td>
<td>Flame ionization detector</td>
</tr>
<tr>
<td>GAP</td>
<td>Good agricultural practice</td>
</tr>
<tr>
<td>GC</td>
<td>Gas chromatography</td>
</tr>
<tr>
<td>GC/MS</td>
<td>Gas chromatography /mass spectrometry</td>
</tr>
<tr>
<td>GSH-PX</td>
<td>Glutathione peroxidase</td>
</tr>
<tr>
<td>h</td>
<td>Hour(s)</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
</tbody>
</table>
HP-β-CD  Hydroxypropyl-β-cyclodextrin
HLB  Hydrophile lipophile balance
HSCCC  High-speed counter-current chromatography
5-HT  5-hydroxytryptamine
IAS  Initial average speed
i.g.  Intragastrical injection
i.p.  Intraperitoneal injection
IR  Infrared spectrum
JNK  c-Jun N-terminal protein kinase
LD<sub>50</sub>  Median lethal dose
LIG  Ligustilide
LLE  Liquid-liquid extraction
MDA  Malondialdehyde
ME  Micro-emulsion
Min  Minute(s)
NA  Noradrenaline
NMR  Nuclear Magnetic Resonance
PCC  Pressurized column chromatography
PG  Propylene glycol
PGF<sub>2α</sub>  Prostaglandin F2alpha
PLE  Pressurized liquid extraction
POM  Polarizing optical microscopy
PRD  Pressure reducing distillation
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>P-TsOH</td>
<td>Methyl toluenesulfonate</td>
</tr>
<tr>
<td>RH</td>
<td>Relative humidity</td>
</tr>
<tr>
<td>ROCC</td>
<td>Receptor-operated calcium channel</td>
</tr>
<tr>
<td>RSD</td>
<td>Relative standard deviation</td>
</tr>
<tr>
<td>rt</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SFE</td>
<td>Supercritical fluid extraction</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>TCM</td>
<td>Traditional Chinese medicine</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>TTC</td>
<td>Tetrazolium chloride</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet spectrum</td>
</tr>
<tr>
<td>VDCC</td>
<td>Voltage-dependent calcium channel</td>
</tr>
<tr>
<td>VSMCs</td>
<td>Vascular smooth muscle cells</td>
</tr>
<tr>
<td>VLC</td>
<td>Vacuum liquid chromatography</td>
</tr>
<tr>
<td>Z-LIG</td>
<td>Z-ligustilide</td>
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</tbody>
</table>
CHAPTER 1

INTRODUCTION

1.1 INTRODUCTORY STATEMENT

The aim of this chapter is to provide a general introduction to this thesis.

Traditional Chinese Medicine (TCM) is the precious inheritance of the Chinese culture. It is also the material foundation for Chinese doctors to treat diseases. There are a lot of compounds with different treatment effects for various diseases. Therefore TCM is a fertile ground for new compounds with good pharmacology effect and can perhaps prevent and treat serious diseases, such as cerebrovascular disease, Alzheimer’s disease and so on. However, because of the low level of the technique and process development, TCM has not been developed and used sufficiently. Therefore, the low level of technique and process restricts the development of the new medicine and the modernization of TCM.

Angelica sinensis (当归) (Lin et al., 1979) and Ligusticum chuanxiong (川芎) (Naito et al., 1996) have been used to treat cardiovascular and cerebrovascular diseases in TCM for a long time. Their effects include the enrichment of blood, relief of pain, activation of vital energy and blood circulation and so on. From previous reports, it was found that
their volatile oil plays a main role in treating diseases. And the 3-alkylphthalide derivatives are the most effective parts of the pharmacology activity in the volatile oil. Modern phytochemistry studies showed that 3-n-butylidene-4,5-dihydrophthalide (ligustilide) is the main 3-alkylphthalide derivative and lipophilic component in their volatile oil. Ligustilide (LIG) in the raw medicinal material has two configurations, Z-ligustilide (Z-LIG) and E-ligustilide (E-LIG) (structures shown in Fig 1-1). Z-LIG, which is much more stable than E-LIG base on the superior structure, is 10 times much more than the E-LIG in volatile oil of Angelicae sinensis and Ligusticum chuanxiong (Li et al., 2001). Thus, Z-LIG is the main configuration and the key subject of LIG to study with. There are also other 3-alkylphthalide derivatives in the volatile oil, which include butylidene-phthalide (Fig1-2, 1 and 2), 3-n-butylphthalid (Fig1-2, 3), senkyunolide (Fig1-2, 4) and so on, with a different percentage of different components in it.

LIG is a characteristic component of the 3-alkylphthalide derivatives and has been considered as the main biologically active component of many important medical plants, such as Angelica sinensis (Zhao et al., 2003), Ligusticum chuangxiong (Wang, 2003), Ligusticum wallichii (Wang et al., 1984) and Cnidium officinale (Bohrmann et al., 1967). LIG has a chemical structure similar to 3-n-butylphthalide (Liu and Feng, 1995), except the different active dihydrobenzene and 3-double bond, which is related to the antioxidant activity. Since the involvement of oxidative stress in neuronal loss following ischemia is well established (Li, et al., 1995; Ratan et al., 1994; Whittemore et al., 1995), the structural feature of LIG suggests that it is worth developing with good pharmacology effect.
The study of LIG has a history of more than 40 years. Researchers in recent years have discovered that LIG is a compound with a good development prospect. Local and foreign development institutes are paying more attention to LIG. Now China has already established the Good Agricultural Practice (GAP) cultivating base for *Angelica sinensis* and *Ligusticum chuanxiong*. It was reported that the content of LIG is about 1% in both *Angelicae sinensis* (Zhao et al., 2003; Li et al., 2001) and *Ligusticum chuangxiong* (Wang, 2003). They provide abundant resources for developing LIG in China. If the basic study of LIG was enlarged, and the preparation and stability problems are solved with new technical methods, it would be possible to develop LIG into new drugs. This will not only improve the international competitiveness of medicines and chemical reagents, but also be a great development in medicine industry in China as well. This type of innovated natural medicine will make tremendous contribution to medicine history as well.

Because of its special chemical structure and instability, the preparation and separation of LIG are very difficult. The preservation of high-purity LIG is also difficult. Therefore, the development of LIG research is enormously limited. Research for the preparation and separation of LIG is still in the laboratory level (Hu and Ding, 2003; Chen and Xue, 2003). And there are not any reports on the industrial separation of LIG. The systemic stability research and pharmaceutical preparation of LIG are also not reported.

**1.2 PRIMARY PROPERTIES OF LIGUSTILIDE (LIG)**
LIG is a lipophilic compound, lightly yellow in color, with a molecular weight of 190, and was soluble in organic solvents, ethanol, dimethl sulphoxide (DMSO), ethyl acetate etc. It was firstly isolated from the roots of *Ligusticum acutilobum* of Umbelliferae plant in 1960 (Mitusuhashi et al., 1960), and its structure was identified the next year (Mitusuhashi et al., 1961).

The CA index name of LIG is 1(3H)-Isobenzofuranone, 3-butylidene-4,5-dihydro- (9CI), and the chemical names are 3-butylidene-4, 5-dihydrophtalide, 1,5-cyclohexadiene-1-carboxylic acid, 2-(1-hydroxy-1-pentenyl)-, lactone(7CI) and phthalide,3- butylidene-4,5-dihydro- (8CI). The structure of LIG consists of double bonds in the ring outside. There is a very unstable factor in chemical structure of LIG. The structure is a typical structure of phthalide, and there is an active butenyl group on the third position. At room temperature, LIG is an unstable liquid compound due to the active dihydrobenzene, which could be changed into other phthalides through oxidation, isomerization, dimerization, etc. Because of the instability, the preparation and preservation of LIG are very difficult.

1.3 PHARMACOLOGY OF LIGUSTILIDE

3-alkylphthalide derivatives, which exist widely in natural Umbelliferaeaceae plants, had been shown to possess various bioactive effects. 3-n-butyrophthalide is a characteristic component of 3-alkylphthalide derivatives. Recent studies had shown that 3-n-butyrophthalide, a component isolated from the seeds of *Apium graveolens* Linn, had
many significant protective effects on brain ischemic damage, including reducing the
neuronal apoptosis and the infarct volume in transient focal cerebral ischemia in rats,
prolonging life span and improving the neurological deficit in stroke-prone and
spontaneously hypertensive rats (Chang and Wang, 2003; Liu and Feng, 1995; Zhang and
Feng, 1996). This agent had been proven effective in clinical trials and approved for the
treatment of acute ischemic stroke by the State Food and Drug Administration of China
(Cui et al., 2005; Wang and Huang, 2003).

LIG is the other typical compound of 3-alkylphthalide derivatives. Many studies have
reported that LIG has strong pharmacological effect on cardio-cerebral vessels, the
central nervous system, the circulatory system and so on, and could be used to prevent
and treat microcirculation disturbance such as shock, diabetes complication,
cardiorespiratory function disturbance, cerebral function disturbance and multiorgan
failure. Several patents (Teng et al., 2007; Yang et al., 2004; Teng et al., 2006; Qian et al.,
2005) have been applied for the application of LIG to prevent and treat cardiovascular
and cerebrovascular diseases such as atherosclerosis and cerebral ischemia disease.

1.3.1 Effect on Cardiovascular and Cerebrovascular System

1.3.1.1 Effect on Cardiovascular System

It has been reported that LIG inhibits vascular smooth muscle cells proliferation (Lu et al.,
2006). It is believed that the proliferation and migration of vascular smooth muscle cells
(VSMCs) lead to atherosclerosis and venous bypass graft disease. LIG is widely used to treat some pathobiology such as atherosclerosis and hypertension. Results showed that LIG significantly inhibits VSMCs proliferation and cell cycle progression. Further analysis showed that LIG suppresses reactive oxygen species production and extracellular signal-related kinases (ERK), c-Jun N-terminal protein kinase (JNK) and p38 MAP kinase. Cells were treated with antioxidant, superoxide dismutase, catalase, and DPI, respectively, leading to repressed ERK, JNK, and p38 activation. The inhibitors of mitogen activated protein kinase (MAPK), PD98059, SB203580 and Sp600125 inhibit cell proliferation. These findings suggest that the antiproliferative effect of LIG is associated with the decrement of reactive oxygen species which results in the suppression of the MAPK pathway. Thus, LIG is an effective agent in preventing cardiovascular diseases (Lu et al., 2006).

Cao et al. found that LIG induces vasodilatation in the rat mesenteric artery by inhibiting the voltage-dependent calcium channel (VDCC) and receptor-operated calcium channel (ROCC), and receptor-mediated Ca^{2+} influx and release (Cao et al, 2006). Their results showed that LIG at a concentration higher than 10μM relaxes the potassium chloride (KCl)-preconstricted rat mesenteric artery in a concentration-dependent manner. The vasodilatation effect of LIG is not dependent on endothelium. LIG concentration-response curves shifting to rightwards be induced by KCl, calcium chloride (CaCl_2), noradrenaline (NA) or 5-hydroxytryptamine (5-HT) in a non-parallel manner. This suggests that the vasodilatation effects are most likely via the VDCC and ROCC. Propranolol, glibenclamide, tetraethylammonium and barium chloride do not affect the
vasodilation induced by LIG, showing that adrenoceptor, ATP sensitive potassium channel, calcium-activated potassium channel and inwardly rectifying potassium channel are not involved in the vasodilatation. LIG concentration-dependently inhibits the vasoconstriction induced by NA or CaCl₂ in Ca²⁺-free medium, indicating that the vasodilatation relates to the inhibition of extracellular Ca²⁺ influx through the VDCC and ROCC, and intracellular Ca²⁺ release from Ca²⁺ storing site. Since the caffeine-induced contraction is inhibited by LIG, the inhibition of intracellular Ca²⁺ released by LIG occurs via the ryanodine receptors (Cao et al, 2006).

Chan et al. studied the vasorelaxation effect of LIG and its underlying mechanism in rat isolated aorta (Chan et al., 2007). LIG has relaxation potency against contractions to 9,11-dideoxy-9α, 11α-methanoepoxyprostaglandin F₂₀, phenylephrine, 5-hydroxytryptamine and KCl. Its vasorelaxation effects are not affected by endothelium removal, the adenylate cyclase inhibitor 9-(tetrahydro-2-furanyl)-9H-purin-6-amine, the guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one or the non-selective K⁺ channel blocker tetraethylammonium. This is the first report to demonstrate the vasorelaxation activities of LIG in contractions to various contractile agents in rat isolated aorta. The underlying mechanisms await further investigations.

1.3.1.2 Effect on Cerebrovascular System

The patent (Qian et al. 2006) had reported LIG can prevent and treat ischemic diseases comprising cerebral infarction, nerve deficiency and vascular dementia caused by cerebral ischemia. The therapeutically effective dose (ED) of LIG is 0.1-500 mg/kg/d.
The patent (Xiao et al. 2005) had reported that LIG can treat ischemic heart disease. It was proved by pharmacology reports that LIG has obvious protective effects on myocardial ischemia reperfusion injury in anesthetized rats, and can improve the anti-anoxia ability of mice.

It was reported that LIG could significantly protect the brain from damage induced by transient forebrain cerebral ischemia (Kuang et al. 2006). Transient forebrain cerebral ischemia (FCI) was induced by the bilateral common carotid arteries occlusion for 30 min. LIG was i.p. injected to ICR mice at the beginning of reperfusion. As detected via 2,3,5-tri-Ph tetrazolium chloride (TTC) staining at 24 h following ischemia, the infarction volume in the FCI mice treated without LIG (22.1 ± 2.6%) was significantly larger than that in the FCI mice treated with 5 mg/kg (11.8 ± 5.2%) and 20 mg/kg (2.60 ± 1.5%) LIG (P<0.05 or P<0.01). LIG treatment significantly decreased the level of malondialdehyde (MDA) and increased the activities of the antioxidant enzyme glutathione peroxidase (GSH-PX) and superoxide dismutase (SOD) in the ischemic brain tissues (P < 0.05 or P < 0.01 vs. FCI group). In addition, LIG provided a great increase in Bcl-2 expression as well as a significant decrease in Bax and caspase-3 immunoreactivities in the ischemic cortex. The findings demonstrated that LIG could protect ischemia/reperfusion-induced brain injury by minimizing oxidative stress and anti-apoptosis.

1.3.2 Effect on Central Nervous System (CNS)
It was reported that the antioxidant and anti-apoptotic properties of LIG may contribute to the neuroprotective potential of LIG in cerebral ischemic damage (Kuang et al. 2006; Peng et al, 2007). Xie and Tao found that LIG exhibited mild inhibitory effect in the nervous system, which suggested that LIG was probably one of the active components of the central nervous inhibition of *Radix Angelica sinensis* (Xie and Tao, 1985). Their results showed that LIG (98 mg/kg, i.p.) significantly decreased the spontaneous movement of mice; 98 or 196 mg/kg (i.p.) of LIG antagonized the central excitation reaction induced by ketamine in mice, shortening the latency of mice sleep and the lasting time induced by the pentobarbital and decreased body temperature of mice from 37.9 ± 0.2°C and 38.1 ± 0.2°C to 36.8 ± 0.4°C and 35.9 ± 0.2°C, respectively; 294 mg/kg LIG (i.p.) inhibited the electric-induced irritating reaction of the mice. In addition, Matsumoto et al. found that intraperitoneal injection of LIG (5-20 mg/kg) or butylidene-phthalide (10-30 mg/kg) could reverse the pentobarbital sleep decrease in isolated mice, and both components (20mg/kg, i.p.) attenuated the suppressive effects of yohimbine, methoxamine and a benzodiazepine inverse agonist, FG7142, on the pentobarbital sleep in some group-housed mice. These results suggested that the central noradrenergic and/or GABA systems were implicated in the effects of these components (Matsumoto et al., 1998).

Juice squeezed from fresh celery leaves has long been used in China for the treatment of epilepsy. Two anticonvulsive compounds, butylphthalide and LIG, were isolated from the seeds of the plant *Apium graveolens* (Yu and You, 1984). Yu and You reported that LIG produced protection action against maximal electroshock seizure test, minimal
electroshock threshold test, metrazol seizure threshold test and maximal audiogenic seizure in mice and rats. Kobayashi et al.(1989) reported that LIG was used as central nervous system tranquilizers.

1.3.3 Relaxing Effect on Muscle

1.3.3.1. Effect on Smooth Muscle

According to a study by Tao et al. (1984), LIG had a remarkable antiasthmatic effect in vivo and in vitro experiment. When injected into guinea pigs intraperitoneally at a dose of 0.14 ml/kg, the asthmatic reaction induced by acetylcholine and histamine was immediately impeded with potency approximately equal to the action of aminophylline (50 mg/kg). Lung overflow experiment showed that an intravenous injection of LIG (0.08 ml/kg) into the anesthetic guinea pig caused a complete or partial block of the reaction of histamine at the dose of 2-10 µg/kg. LIG also exhibited an antispasmodic effect on isolated trachea strip of the guinea pig contracted by acetylcholine, histamine and barium chloride, and a relaxation effect on trachea strip under normal tension. Addition of propranolol did not affect these actions (Tao et al. 1984).

It was reported that LIG inhibited the contraction of rat uterus smooth muscle (Du et al. 2007). In isolated rat uterine, LIG inhibited the spontaneous periodic contraction in a concentration-dependent manner, and attenuated prostaglandin F2alpha (PGF2α) or acetylcholine chloride (Ach)-induced uterine contractions. It was also observed that LIG
affected significantly the oxytocin-induced increase in the contraction of uterine horns that were incubated not only in the Locke soln, but also in the Ca\textsuperscript{2+}-free solvent. The results clearly showed that LIG had multiple effects on the uterine smooth muscles, suggesting that LIG possesses a non-specific antispasmodic function. The data also implied strongly that LIG had the potential to be developed into an effective drug for the prevention and treatment of primary dysmenorrheal (Qian et al., 2006) and other gynecological diseases (Zhao, 2003).

1.3.3.2 Effect on Skeletal Muscle

Three phthalide compounds (LIG, cnidilide and senkyunolide) were extracted from the rhizome of \textit{Cnidium officinale}, and the centrally acting muscle-relaxant effect was investigated on the crossed extensor reflex in anesthetized rats. The results showed that these 3 compounds (administered i.p.) could depress the reflex response. Their depressive potencies were almost the same, and their potencies were also the same or somewhat weaker than that of mephenesin. Since no curare-like action was observed, the muscle relaxation induced by these phthalide compounds was considered to be due to a central action (Ozaki et al., 1989).

1.3.4 Regulation on Microcirculation

The invention (Liu et al., 2004) provided the use of LIG for treating and preventing the
microcirculation disorder related diseases. LIG is a main active constituent in the essential oil from the rhizome of *Ligusticum chuanxiong*. The influence of the essential oil on microcirculation in conjunctiva bulbar was compared in rabbits intravenously injected with dextran T<sub>500</sub> before and after the LIG was decomposed. Results showed that there was no difference between the two types of essential oil in the spasmolysis of venule. But the effects of the LIG-decomposed essential oil were obviously weakened in spasmolysis of arteriole, decoagulating erythrocyte, increasing the number of opened capillary, accelerating the speed of blood flow and enlarging the amount of blood flow in microcirculation (Shi et al., 1995). Thus, LIG is probably an active constituent to improve microcirculation.

1.3.5 Effect on Anti-inflammatory

Beck et al. had reported that LIG had weak antiviral properties and a weak antimicrobial activity against Gram-positive, Gram-negative and yeast microorganisms. The broad biological activity of LIG and its electrophilic reactivity are consistent with the use of Ligusticum species in folk medicine (Beck et al., 1995). It was reported the studied of anti-inflammatory, analgesic and antipyretic effects of LIG (Yao et al., 2006). The effects of LIG on acute and chronic phases of inflammation were studied in carrageenan- and dextran-induced paw edema and cotton pellet-induced granuloma, respectively. Antipyretic activity of LIG was evaluated in a yeast-induced hyperpyrexia model in rats. In the anti-inflammatory studies, LIG showed a significant dose-dependent inhibition of 22.2%, 49.4% and 76.5% and 20.8%, 44.2% and 75.3% in carrageenan- and
dextran-induced paw edema, and 29.2%, 44.9% and 58.8% in cotton pellet-induced granuloma, respectively (P<0.001). LIG also exhibited a significant dose-dependent inhibition on peritoneal leukocyte migration in mice at the inhibition rate of 20.7, 35.6 and 48.2%. Oral administration of LIG significantly reduced the elevated temperature in rats (P<0.001). The results suggest that LIG possesses potent anti-inflammatory and analgesic activities (Yao et al., 2006; Heimbegner et al., 2004).

1.3.6 Insecticidal Effect

The insecticidal activity of two phthalides from *Angelica acutiloba* was investigated and compared with that of rotenone (Miyazawa et al., 2004). Z-butylidene-phthalide and LIG exhibited LC50 values of 0.94, 2.54, µmol/mL respectively of a diet concentration against larvae of *D. melanogaster*. Against both sexes (males/females, 1:1) of adults (5-7 days old), Z-butylidene-phthalide showed the most potent activity with a LD50 value of 0.84 µg/adult. Z-butylidene-phthalide is a more active insecticide than rotenone (LD50 = 3.68 µg/adult) and has the potential as a novel insect control agent. However, LIG was inactive against adults. The structure-activity relationship of phthalides isolated indicated that the aromaticity appeared to play an important role in the activity of both larvae and adults. It was reported that preliminary bioassays indicated LIG contained selective phytotoxic activity against monocots and antifungal activity against *Colletotrichum fragariae* (Meepagala et al., 2005)

1.4 RESOURCE OF LIGUSTILIDE
1.4.1 Plant Resource of LIG

LIG is the active ingredient of *Ligustrum* and *Angelicae* plant species in *Umbelliferae* which are widely distributed in Asia, North America and Europe. The first isolation of LIG was from *Ligusticum Acutilobum* in 1960 (Mitsuhashi et al., 1960), following, from *Cnidium officinale* Makino in 1977 (Yamagishi et al., 1977), *Angelica sinensis* (Oliv.) Diels (Lin et al., 1979) and *Levisticum officinale* Koch (Fang et al., 1979). Most of these plants are used as medicines among the people. The content of LIG is the highest in those plants in Asia, especially in China. A lot of studies have shown that LIG is actually a typical phthalide component of numerous *Umbelliferae* plants and has been considered as the main biologically active component of many important medical plants, such as *Angelica sinensis* (Lin et al., 1979), *Ligusticum wallichii* (Wang et al., 1984), *Ligusticum chuangxiong* (Naito et al., 1996) and *Cnidium officinale* (Bohrmann et al., 1967). It was also reported that the content of LIG reached up to 1% of *Angelica sinensis* growing in Min county, Gansu province, Good Agricultural Practice (GAP) cultivating base and 0.5% in Sichuan, Yunnan, Shanxi province, much more than in the same genus plants in Japan(0.1-0.15%) and Korea(0.3%) (Zhao et al., 2003). It was also reported that the content of LIG is about 1% in *Ligusticum chuangxiong* growing in Dujiangyan city, Sichuan province, GAP cultivating base, much higher than that in the same genus of *Ligusticum chuangxiong* in Japan (Wang, 2003). The roots of *Angelica sinensis* is the more important available source of natural LIG for over 50% of its essential oil comprises of LIG (Fang et al., 1979; Li et al., 2001). The plant resource for LIG is very
abundant in China for its research and development.

1.4.2 Synthesis of LIG

The LIG content in traditional Chinese medicine is very high, and the output of its raw material is suitable and could satisfy the need of industrialization production. But the nature couldn’t always offer resources without limit, no matter in the now or future. The synthesis of LIG has also drawn more attention. Li et al. reported a detailed synthesis of LIG with four steps (Li et al., 1994). In this synthesis, the preparation of LIG comprised the reaction between o-carboxybenzaldehyde and KOCMe3 with butyltriphenyl phosphonium bromide to give a cis-trans mixture of o-(1-penten-1-yl) benzoic acid, the oxidation with peracetic acid to give a mixture of threo- and erythro-3-(1-hydroxybutyl) phthalide, the reduction with Na-NH3 to give a mixture of threo- and erythro-4,5-dihydro -3-(1-hydroxybutyl)phthalide, and the subsequent reaction at room temperature to give LIG. But the synthesis yield rate of LIG was only about 5% (Li et al., 1994). It was also reported that the synthesis of naturally occurred LIG was accomplished by the cyclization of -1-pentynylbenzoic acid, which was prepared from 1,4-cyclohexanedione monoethylene ketal in several steps, and then catalyzed by silver iodide or silver in a mild reaction condition as a key step (Ogawa et al., 1995). The synthesis yield rate was low as well. Also the synthesis process of LIG was complicated, and the instable problem of LIG had great influence to the product yield. In on word, it still had a long way to go to enhance the LIG yield and thus industrialize its production.
1.5 EXTRACTION AND ISOLATION OF LIGUSTILIDE

1.5.1 Extraction of LIG

Since the LIG was one of the volatile components, so extraction of the volatile oil from plant was the first step to extract the LIG. It was reported that the volatile oil containing of LIG was extracted by steam distillation (Hu et al., 2003; Choudhury et al., 2000). Mitsuhashi and Kobayashi reported that the volatile oil containing of LIG was extracted by solvent extraction with hexane (Mitsuhashi et al., 1960), ethanol (Kobayashi et al., 1989) and acetone (Kanamori et al., 1996). It was reported that the extraction process of LIG in *Angelica sinensis* and *Ligusticum chuanxiong* was optimized. HPLC assay was established to detect the content of LIG in the extracted solvent and the extraction process of LIG with ethanol was studied and optimized by the orthogonal design. The optimum extraction process was as follows: adding 8-times of 85% ethanol to the sample for extracted, refluxing and extracting 3 times, 1.0 h each time (Hu et al., 2005). There have many reports said that the volatile oil contained of LIG always extracted by supercritical fluid extraction (SFE). (Huang et al., 2002; luo et al., 2006; Wu et al., 1998; Ge et al., 2000).

Some common extraction methods of volatile oil include steam distillation, supercritical fluid extraction and organic solvent diffusion. All of them have their own advantages and disadvantages. Extraction of essential oil from *Angelica sinensis* by SFE was compared with that by steam distillation. The components of the essential oil and content of LIG
extracted by both extraction methods were the same, but the yield of SFE was twice as much as that by steam distillation. Extraction of the essential oil from *Angelica sinensis* by SFE is more satisfactory than that by steam distillation (Li et al., 2001). In this report, the condition of SFE for the extraction of volatile oil in *Ligusticum chuanxiong* was studied and its extraction rate was compared with that by steam distillation. A five-factor and four-level orthogonal test was used to optimize the SFE conditions. The result showed the many advantages of the SFE method: short extraction time and high extraction efficiency, etc (Yuan et al., 2000). Hu and Ding reported that the essential oil of *Angelica sinensis* was extracted by SFE, steam distillation and solvent extraction. The results showed that the SFE method was the best (Hu et al., 2003).

Although the cost of steam distillation is low, the high abstraction temperature, long warm-up time (usually from 5-14 hours) and the thermal instability of LIG cause a low content of abstracted LIG. The advantages of SFE include low abstraction temperature, complete extraction, the composition of volatile oil was not easily being volatile, having no change in high temperature and simple manipulation, but its extraction cost is high, which especially limited the production in industrial scale. Organic solvent diffusion is widely used because its extraction is simple and complete, and there is only a little change of the LIG and the cost is low. Ethanol, which is a commonly used organic solvent, extracts a large amount of substance of great polarity, such as sucrose, etc, so that the impurity needed to be disposed elaborately to obtain a pure extracted LIG.

**1.5.2 Isolation of LIG**
LIG was isolated from the ethanol extraction of the roots of *Angelica sinensis* by the chromatography method (Lu et al., 2003). Naito et al. reported that LIG was isolated and purified with CHCl$_3$-MeOH-H$_2$O and n-hexane-MeOH-H$_2$O by chromatography using Celite and silica gel (Natio et al., 1993). *Angelica acutiloba* was extracted with 2:1 hexane-Et$_2$O, then 1:1 CHCl$_3$-MeOH, the extractions partitioned in CHCl$_3$-MeOH-H$_2$O which CHCl$_3$ was combined with the previous hexane-Et$_2$O, re-partitioned, and then the solvent was distilled to give LIG. Similarly 5 other fractions were also isolated and identified (Kobayashi et al., 1989).

Zhang et al. reported that high-speed counter-current chromatography (HSCCC) was used in the isolation of LIG in his study. Different solvent systems for HSCCC were compared. A system composed of hexane-Et acetate- methanol-water-acetonitrile in the ratio of 8:2:5:5:3 (v/v) was found to be the optimum for HSCCC of the essential oil. LIG and senkyunolide A were separated by HSCCC with a purity of 98% which detected by GC (Zhang et al., 2006).

The pressurized liquid extraction (PLE) was also used in isolation of LIG and reported by Li (Li et al. in 2006). PLE, one of the most promising and recent sample preparation techniques, offers the advantages of reducing solvent consumption and allowing for automated sample handling. It is being exploited in diverse areas because of its distinct advantages. However, because the extraction is performed at elevated temperatures using PLE, thermal degradation could be a concern. In this study, researchers carried out a comparative study to evaluate PLE as a possible alternative to current extraction methods like Soxhlet and sonication for the simultaneous extraction of LIG,
Z-butylidene-phthalide and ferulic acid in *Angelica sinensis*. The operating parameters for PLE including extraction solvent, particle size, pressure, temperature, static extraction time, flush volume and numbers of extraction were optimized by using a univariate approach coupled with a central composite design (CCD) in order to obtain the highest extraction efficiency. The results showed that PLE was a simple, highly efficient and automated method with lower solvent consumption compared to conventional extraction methods such as Soxhlet and sonication. PLE could be used for the simultaneous extraction of LIG, Z-butylidene-phthalide and ferulic acid in *Angelica sinensis* (Li et al., 2006).

It was reported that silicic acid was used as carrier and chloroform and N-hexane-Dichloromethane as solvent to prepare LIG. In addition, LIG could be made with fractionation (Mitsuhashi et al., 1960; Bohrmann et al., 1967). Zhao (Zhao, 2003) reported that hexanol was used to extract the volatile oil from *Angelica sinensis*, and dissolved the volatile oil in petroleum ether, 5%NaOH was added to remove phenol oil and acid-stage oil, then the LIG was extracted by silica gel column chromatography. Chloroform was used as solvent for elution and the fractionation was made twice at temperature between 168-169°C to obtain LIG. It was reported that LIG could also be made with vacuum fractionation (Mitsuhashi et al., 1968).

It was reported in many literatures that silica gel was used as a carrier for the isolation of LIG in the volatile oil. Some made thin layer chromatography for isolation, most isolated LIG with column chromatography. The used elution solvents included petroleum
ether-ethyl acetate, petroleum ether-ether, n-hexane-ether, n-hexane-acetone and n-hexane-chloroform. These solvents were mixed together with different proportion for isocratic or gradient elution. Since LIG had low polarity, any one of the two solvents with low polarity must be used for isolation.

Currently, LIG was prepared at laboratory level. There had not report said that the isolation method of LIG had the advantages of low toxicity, safe to use, high yields, and high purity, and was suitable for industrial production. The isolated content of LIG only reached milligram level, however it must increased to gram level and produced industrially for research and develop thoroughly. These were the emphasis and key point in our project.

1.6 DETERMINATION OF LIGUSTILIDE

1.6.1 Thin Layer Chromatography (TLC)

It was reported that LIG was detected in the extractions of *Angelica sinensis*, *Levisticum officinale*, *Ligusticum jeholense*, *Ligusticum acutifolium*, *Ligusticum chuanxiong*, *Angelica dohuzica* and *Arehangelica officinalis* by TLC. LIG was chromatographed on silica gel G plates using CCl₄-xylene-CHCl₃-hexane (4:3:2:0.5) as eluent. After spraying 1% vanillin, the spot of LIG was detected at 365 nm (Lu et al., 1980). Wang et al. reported that a method for detecting LIG in *Angelica sinensis* was developed using dual wavelength TLC-scanning (λ s = 290 nm, λ R =370 nm) and the developing solvent was
consisted of petroleum ether-EtOAc (9.5:0.5) (Wang et al., 2003).

1.6.2 High Performance Liquid Chromatography (HPLC)

It was reported that a reversed-phase HPLC method was developed for the simultaneous detection of four main lactone components: senkyunolide-H, senkyunolide-I, sedanenolide and LIG in *Ligusticum chuanxiong*. Samples were pretreated using ultrasonic extraction and 80% ethanol was used as extractant in the experiment. Chromatography analysis was carried out using an XDB-C₈ column and the mobile phase consisted of methanol-1% acetic acid water (55:45→100:0, v/v, in 15 min). The flow rate was 0.8 mL/min. A diode array detector was used to detect the compounds and 280 nm was chosen as the detection wavelength. The whole process could be performed within 15 min. The recovery of the four lactone components was in the range of 96%-108%. This rapid and accurate method has been successfully applied to the simultaneous detection of the four lactone components in *Ligusticum chuanxiong* (Cao et al., 2005; Ding et al., 2004). An HPLC method to detect LIG from *Ligusticum chuanxiong* was studied. The column was Luna silica (150mm×4.6mm ID); the mobile phase consisted of n-hexane-Et acetate-chloroform; the flow rate was 0.8 mL/min and the UV detector was set at 320 nm. The method displayed good linearity within 0.1-2.0 µg. The average recovery was 100.7% (Chao, 2004).

The determination of LIG in volatile oil from *Ligusticum chuanxiong* with RP-HPLC was
studied. ODS$_2$ column (4.6mm×200mm, 5 µm) was used and nitrendipine was used as the internal standard. The mobile phase consisted of methanol, acetonitrile and water (33:21:46). The LIG was at 275 nm. The linear range was 2.92-29.2 mg/L for LIG. The average recovery of LIG was 95.1% with relative standard deviation (RSD) 2.3%. The method was simple and could be used to detect LIG with satisfactory accuracy and reproducibility (Lin et al., 2004). LIG was analyzed by HPLC with UV absorbance detection, and the conditions were: Nova-Pak C18 column with naphthalene as the internal standard, methanol-10% isopropyl alcohol solution (53:47 v/v) as mobile phase with a flow rate of 0.8 mL/min, and the detection wavelength was 280 nm. The recovery and RSD were achieved for solid-liquid trap and solvent trap as 99.34%, 1.70% vs. 92.11%, 5.72%, respectively (Wu et al., 1998).

High-performance liquid chromatographic separation of LIG in the Angelica plants was studied. A Nucleosil 100-3C$_{18}$ column of 4.6 mm × 25 cm could be used to give a complete separation of LIG and internal standard pyrene from impurities in plant extractions using 75% acetonitrile mobile phase and a fluorometric detector. The excitation wavelength of the detector was fixed at 365 nm and total fluorescence produced from a column effluent was measured to prevent a diffused and/or scattered exciting light. A highly sensitive and reliable analysis value was obtained by this minor revision of the detector. Calibration curves, LIG concentration versus LIG-pyrene peak-area ratio, were detected over the two working ranges with their 95% fiducial limits of determination. As an application of this analysis method, LIG contents in five Angelica Radix and several parts of the Angelica plants (TOHKI, an oriental crude drug) were
LIG and butyldene-phthalide were detected in several samples of Angelica root and Cnidium rhizomes by high-performance liquid chromatography. A powder sample was extracted with hexane at 40-50°C for 30 min. The hexane solvent was analyzed on a high-performance liquid chromatography with Unisil CP 5µm column, hexane-CH$_2$Cl$_2$ (95:5) solvent, 1.5 ml/min flow rate and detection at 240nm (Yamagish, 1982).

1.6.3 Gas Chromatography (GC)

It was reported that a GC method was developed for the determination of LIG in the essential oil of *Angelica sinensis*. The chromatography conditions were as follows: SE-54 capillary column (50 m × 0.2 mm), column temperature 240°C, column head pressure 210 kPa, vaporizer temperature 280°C, FID detector temperature 280°C, split ratio 30:1, flow rate 40 mL/min and injection volume 0.5 µl. The linear range of LIG was 9.12-21.28 mg/mL, with a correlation coefficient (r) of 0.9999. The average recovery of the added sample was 98.96%, with RSD of 1.89% (n=6). The method is precise, reproducible and stable (Li et al., 2005).

A capillary gas chromatography method for the determination of LIG was proposed hoping to optimize conditions for the supercritical fluid extraction of LIG. The extractions were separated by a SE-54 column with a second order programmed temperature. LIG was quantitatively analyzed by using octadecane as an internal standard. There was a good linear relationship between the peak area and the concentration of LIG in the range
of 0.7-1.8 µg/L. A RSD of 2.2% (n = 6) and recoveries of 96 ± 2 % were obtained (Huang et al., 2002).

A GC-MS method for the determination of LIG in the *Angelica sinensis* oil was developed. The carrier gas was helium. The injector temperature was 250°C and the detector temperature was 280°C. LIG was detected by GC-MS with selected ion and qualifier ion monitoring. The standard curve was linear for LIG within 1.34 ng and 1.34 pg and the sensitivity was 0.1 ng/ml. The average recovery was 98.9% with RSD 2.2%. This method was sensitive, rapid and accurate (Zhou et al., 2002).

A gas chromatography/mass spectrometry (GC/MS) method was developed to study the pharmacokinetics of LIG following oral administration to rats. The method was used for the analysis of samples taken from rats. The samples were prepared by liquid-liquid extraction (LLE) using an n-hexane-ether (2:1) solvent mixture for a sample clean-up step and analyzed by GC/MS with a quadrupole MS detector in a selected ion monitoring mode (m/z 190). The calibration curves were linear over the concentration range 0.172-8.60 µg/mL (r > 0.99) for blood samples and a different range (r > 0.99) for different tissue samples. The limit of detection (LOD) was 1.0 ng/mL or 1.0 ng/g (3 times the signal-noise ratio). Within- and between-day precision expressed as the RSD for the method was 1.58-3.88 and 2.99-4.91%, respectively. The recovery for all samples was >80%, except for the liver samples (>70%). The main pharmacokinetic parameters obtained were: Tmax = 0.65 ± 0.07 h, Cmax = 1.5 ± 0.2 g/mL, AUC = 34 ± 6 h g/mL and Ka = 3.5± 0.6/h. The results showed that LIG was easily absorbed, but its elimination was
slow, from 3 to 12 h after oral administration. The concentrations of LIG in rat
cerebellum, cerebrum, spleen and kidney were higher than in other organs (Shi et al.,
2006).

1.7 STABILITY OF LIGUSTILIDE

LIG, one of the biologically active components in *Angelica sinensis* and *Ligusticum
chuanxiong*, is an unstable compound and decomposes rapidly at room temperature.
There are many reports which investigate the stability and look for the preservation
condition of LIG. The stability of LIG with solvent effect and its relationship with solvent
effect were studied for the preservation of LIG (Zhou and Li, 2001). LIG was more stable
in cyclohexane and chloroform than in air. The isomerization rate of LIG was remarkably
decreased by 1.6% and 6.7% in cyclohexane and chloroform respectively, and 58% in air.
The results showed that the stability of LIG was greatly improved with the solvent effect,
and keeping LIG in a proper organic solvent helped its preservation (Zhou et al., 2001). It
was also reported that the pure LIG in CHCl_{3} kept in a brown bottle was stable for a year
with or without butylated hydroxyanisole (Hisayuki and Ikunori, 1992).

It was reported that LIG was kept at room temperature, 4°C and -20°C respectively and
examined periodically by GC. The results showed that LIG could be kept only below -20
°C. It was labile to isomerize at room temperature. By GC-MS analysis, it was possible to
trace the structural changes of its isomerization pathway (Li et al., 2000). The stability of
LIG in Fengshikang capsule was also studied by capillary gas chromatography (Chen et
It was reported that a suitable vehicle of LIG was investigated to predict its shelf-life at 25°C by its degradation laws. Factors including temperature, light, pH value, co-solvents and antioxidants could all influence the stability of LIG, thereinto antioxidants could markedly improve its stability in aqua solvent by almost 35%. The suitable vehicle for LIG contained 1.5% tween-80, 0.3% Vitamin C, and 20% propylene glycol (PG). Furthermore, the degradation rates of LIG were found to conform to a rate equation following the Weibull probability distribution within a range of degradation ratio, and the equation could be expressed as follows: \( \ln \ln \left( \frac{1}{1 - a} \right) = \ln k + m \ln t \). Where \( a \) was the degradation ratio; \( t \) was time; \( m \) and \( k \) were constants relating to the degradation rate. The degradation rate would get greater with the increase of parameter \( k \). According to the degradation law obtained from the equation, the drug shelf-life (10% of active ingredient degraded, T90) in this vehicle was predicted to be more than 1.77 years at 25°C through the Arrhenius equation and accelerating experiments (Cui and Feng, 2006).

Gijbels et al. had reported that the artifact naturally occurring mixtures of phthalides which used to pack the gas chromatography column, was sometimes detected from LIG originally. Active sites of Chromosorb W were responsible for the formation of the artifact, which was assumed to be E-LIG since its mass spectrum was identical with that of LIG. No isomerization was observed when freshly coated Chromosorb W was used or when the support was deactivated by BzCl or melamine (Gijbels et al., 1982).
1.8 OBJECTIVES

This thesis will investigate the isolation, stability, synthesis, pharmaceutical preparation and toxicity of LIG.

1.8.1 Part 1

This study aims to:

1) Investigate the extraction and isolation of LIG in the raw material through different methods, and look for a good separation method for LIG.

2) Investigate the property and instable factors of LIG with high-purity. Then the results will indicate the industrial isolation method for LIG with fast preparation, high production yield and high-purity, and elucidate the physical as well as chemical property and stability influence factor of LIG. These data will provide the information for preservation, reference design and pharmaceutical preparation of LIG.

1.8.2 Part 2

The stability of LIG will be investigated systematically with high-purity LIG, and stabilizer to increase the stability of LIG will be found out. On this basis the LIG reference will be developed, and the LIG reference can be used as the standard for LIG in the industry production and the reference in the raw material. And the chemical synthesis of LIG will be investigated to enlarge the resource of LIG.
1.8.3 Part 3

We will conduct further study on the cyclodextrin (CD) inclusion and microemulsion (ME) of LIG, and establish the process and quality standard of each pharmaceutical preparation and inspect the stability of each preparation. So a solid foundation will be laid in the development of different pharmaceutical preparations of LIG for suitable clinical use. The acute toxicity of LIG will also be investigated. So this study will provide a good foundation for the new drug development of LIG.
Figure 1-1. The configuration structures of LIG.

Figure 1-2. The structures of 3-alkylphthalide
CHAPTER 2

MATERIALS, APPARATUS AND METHODS

2.1 MATERIALS

2.1.1 Reagents and Analysis Kits

Radix *Angelica sinensis*  
Rhizoma *Ligusticum chuanxiong*  
n-Hexane  
95% Ethanol  
Angelica sinensis volatile oil  
Methanol (HPLC grade)  
Isopropyl Alcohol (HPLC grade)  
Anhydrous Ethanol (Analytical grade)  
Anhydrous Ether  
Anhydrous Sodium Sulfate  
Petroleum Ether  
Ethyl Acetate  
Propylene Glycol  
Hydrochloric Acid (HCl)

Meicheng Pharmacy Ltd., China  
Kanghong Pharmacy Ltd., China  
Dai Mo Chemical Co., China  
Dai Mo Chemical Co., China  
Dai Mo Chemical Co., China  
Honeywell International Inc., USA  
Tedia Company, USA  
Bio-Rad Technology Ltd., USA  
Dai Mo Chemical Co., China  
Bei Jing Chemical Co., China  
Dai Mo Chemical Co., China  
Bei Jing Chemical Co., China  
Sigma Chemical Co., USA  
Bei Jing Chemical Co., China
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<tr>
<td>Potassium Chloride</td>
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<tr>
<td>Aluminium Oxide</td>
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<tr>
<td>Magnesium Sulfate</td>
<td>Sigma Chemical Co., USA</td>
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<td>Sigma Chemical Co., USA</td>
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<tr>
<td>Cupric Sulfate</td>
<td>Sigma Chemical Co., USA</td>
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<tr>
<td>Ferrous Sulfate</td>
<td>Sigma Chemical Co., USA</td>
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<td>Zinc Chloride</td>
<td>Sigma Chemical Co., USA</td>
</tr>
<tr>
<td>PEG 400</td>
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<td>Soybean Phosphatide</td>
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<td>Pluronic F68</td>
<td>BASF Chemical Co., Germany</td>
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<td>Soybean oil</td>
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<td>Colza oil</td>
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<td>Silica Gel Plate</td>
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<tr>
<td>Vitamine E</td>
<td>BASF Chemical Co., Germany</td>
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<td>Glycerol</td>
<td>Sigma Chemical Co., USA</td>
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<td>Ascorbic acid</td>
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<td>N-butanol</td>
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<td>BASF Chemical Co., Germany</td>
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<td>Acetonitrile</td>
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### 2.1.2 Apparatus

<table>
<thead>
<tr>
<th>Equipment</th>
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<tr>
<td>HPLC System Agilent 1100 Series</td>
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<tr>
<td>GC-MSD System Agilent 6890/5973</td>
<td>Agilent Technologies, USA</td>
</tr>
<tr>
<td>GC System Agilent 6890N</td>
<td>Agilent Technologies, USA</td>
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<tr>
<td>TLC Numerical Code Photograph System</td>
<td>Camag Caina Co., Switzerland</td>
</tr>
<tr>
<td>SFE Instrument (ISCO Model 260D)</td>
<td>Geyline Ltd., Japan</td>
</tr>
<tr>
<td>Rotary Evaporator (Laborota 4001)</td>
<td>Heidolph Ltd., Germany</td>
</tr>
<tr>
<td>Boiling water bath</td>
<td>Grand Instruments, USA</td>
</tr>
<tr>
<td>Freezer (-80 °C)</td>
<td>Heto Holten AS, Denmark</td>
</tr>
<tr>
<td>Freezer (-20 °C)</td>
<td>Haier Ltd., China</td>
</tr>
</tbody>
</table>
Microcentrifuge
Leica microscope (Model DRIMB)
Microtiter plate reader
pH meter (Model 701 digital)
Rotor–stator homogenizer
Sonicator, Branson Sonifier 250
UV/VIS Spectrometer Lambda35
Water Pump
Vacuum Pump
Membrane-type air pump
NMR Spectrometer Bruker 400MHz
Laser light-Scattering Particle Sizer
High pressure homogenizers
Sociator
Freeze drier
Electron Balance
Ultrasonic Instrument
High Performance Centrifuge
Extracting and Condensing Equipment
Milli-Q water system
Automatic Fraction Collector
Vacuum Fractionating Device
Nicolet AVATAR 360 FT-IR

Eppendorf Ltd., Germany
Leica Ltd., Germany
Bio-Rad Technology Ltd., USA
Orion research Ltd., USA
IKA-Labortechnik, Germany
Branson Inc., USA
PerkinElmer Instruments Ltd., USA
Sibata Scientific Technology Ltd., Japan
Sibata Scientific Technology Ltd., Japan
Sibata Scientific Technology Ltd., Japan
Bruker Optics Co., Germany
Malvern Ltd., USA
Niro Soavi Ltd., Italy
Kun Shan Sonic & Materials Inc., China
Heto Dry Winner Ltd., USA
Sartorius Instruments Ltd., Germany
Shanghai Instrument Co., China
Beckman Coultrur Inc., USA
Yongtai Pharmacy Factory, China
Millipore, Bedford, MA, USA
Shanghai Instrument Co., China
Shanghai Instrument Co., China
Nicolet Vascular Inc., USA
2.1.3 Animals

In this project, mice were used to investigate the acute toxicity of LIG. The use of animals was approved by the Department of Health of Hong Kong, the Animal Ethics Committee of The Hong Kong Polytechnic University and Shenzhen Institute of The Hong Kong Polytechnic University. The mice were supplied by the Animal House of The Hong Kong Polytechnic University or Shenzhen Institute of The Hong Kong Polytechnic University. All the animals were housed in pairs in stainless steel rust-free cages at 21±2°C. The rooms were in a light-dark cycle of 12 hours of light (7:00 to 19:00) and 12 hours of dark (from 19:00 to 7:00). All the animals were fed the Laboratory Rodent Diet (PMI, Brentwood, MO. Catalog# 5001, containing protein 28%, fat 12.1%, fiber 5.3%, Carbohydrate 59.8% and 270 ppm iron) ad libitum and plenty of distilled water was supplied at all times.

2.2 GENERAL METHODS

2.2.1 Method of Chromatography

2.2.1.1 TLC Method

(1) Principle
Thin-layer chromatography (TLC) is the adsorbent or supporter being applied to form a thin layer on flat glass plate, plastic film or aluminum film. After sample application, develop and compare the chromatogram with that prepared by appropriate reference substances under the same conditions for identification, impurities inspection or assay.

(2) Procedure

In the thin layer chromatography (TLC) method, silica gel GF$_{254}$ was used as the coating substance, while hexane-ethyl acetate (10:1) was used as the mobile phase. TLC method was carried out also, and the test solutions were taken in the silica gel plate to develop. Removed the plate and got it dried in the air, then examined under 254nm and 365nm ultraviolet light and dyed with iodin vapor, and then chromatogram was recorded. The position and color of the spots in the chromatogram obtained from the test solutions were compared.

2.2.1.2 HPLC Method

(1) Principle

High Performance Liquid Chromatography (HPLC) is developed from the classical chromatography. By combination of high pressure pump, high efficiency stationary phase and high sensitive detector, HPLC becomes an automatic, fast and high efficiency tool. In HPLC, the mobile phase, a solvent or solvent mixture of suitable polarity (or a buffer solution of suitable ion
strength), is pumped through injector, column which contains the stationary phase, as well as
detector. While the sample is injected into an injector and carried into the column by the mobile
phase, the components are separated on the stationary phase and each component passes through
the detector in succession and a chromatogram is thus recorded.

(2) Procedure

An Agilent 1100 series HPLC system consisting of a vacuum degasser, a quaternary pump, a
100-position autosampler, a thermostated column compartment and a diode array detector (DAD)
(Agilent Technologies, Waldbronn, USA) was used for quantitative analysis and UV spectra
acquisition. For chromatographic analyseis, a Hypersil ODS C18 column (5µm, 150mm × 4.6 mm)
with a compatible guard column (C18, 5µm, 7.5mm × 4.6 mm) was used. The mobile phase
consisted of methanol: 5% isopropyl alcohol (60:40). The mobile phase was filtered through a 0.2
µm filter and degassed by sonication in vacuum for 20 min. The flow rate of the mobile phase was
1 ml/min and the column temperature was 25°C. The LIG peak was detected at an UV absorbance
wavelength of 280 nm. Then the HPLC method was carried out, and 10ul of test solution was
injected respectively into the column, also the chromatogram was determined and recorded. The
recorded time of chromatogram was two times of the retention time of the main peak.

2.2.1.3 GC Method

(1) Principle

In gas chromatography (GC), the mobile phase is an inert gas, known as carrier gas.
And the chromatographic column is packed with an adsorbent, porous polymer beads or a support coated with a liquid phase. The test preparation is injected into the vaporizer with a micro syringe, then vaporized and separated on the stationary phase. Each component passes through the detector in succession and then a chromatogram is thus recorded.

(2) Procedure

An Agilent 6890N GC system (Agilent Technologies, Waldbronn, USA) was used for the analysis instrument. The chromatographic column was HP-5 (5% benzyl methyl polysiloxane, 30m×320um×0.25um). And the detector was the flame ionization detector (FID) with temperature at 250 ℃. Also the inlet temperature was set at 250 ℃. The injection mode was the split with ratio as 50:1. And the oven temperature was 210℃ (8min) and then rose (10℃/Min) to 250℃. Yet the GC method was carried out, and 0.5ul of test solution was injected and examined, then the chromatogram was recorded.

2.2.1.4 GC-MS Method

(1) Principle

The principle of GC-MS is similar to GC, only has the detector different as MS detector. GC is a tool to separate components by different boiling points and reaction between stationary phase and mobile phase. MS can identify compounds by breaking
down their bonding and referring to the database. GC-MS is the combination of above two functions: first separate different compounds and then identify them one by one.

(2) Procedure

An Agilent 6890/5973 GC-MSD system with a quadrupole MS detector in selected ion monitoring mode (Agilent Technologies, Waldbronn, USA) was used for the analysis instrument. The chromatographic column was HP-5ms (5% benzyl methyl polysiloxane, 30m×320um×0.25um). The carrier gas was helium. The injector temperature was 250°C, while the detector temperature was 150 °C(6min) and then rose (10°C/Mim) to 320 °C. The injection mode was the split with ratio as 50:1. GC-MS method was carried out, and 0.5ul of test solution was injected and examined, then the main peak of total ion chromatogram and mass spectrum were recorded.

2.2.1.5 UV Spectrum Method

(1) Principle

The principle of UV is used to measure the absorption of electric wave energy at specific wavelength by molecules. The absorbance is directly proportional to the concentration of substrate being determined. And the electric wave energy is absorbed by the molecule as there is resonance occurred between molecule and the wave energy so wave energy transformed to internal energy of the molecule and cannot reach the UV detector. The detector will translate the received UV light into graphical
and numeral data that presented by the monitor.

(2) Procedure
The instrument, Lambda35 UV/VIS spectrometer (PerkinElmer Instruments, USA) was used for the UV analysis. The sample was taken and dissolved in methanol. The UV spectrometer was switched on and the detecting wavelength of the detector was tuned to proper value, then the spectrometer was warmed up for about 30mins before use. After that, the spectrum and absorbance of the sample were examined and recorded by Lambda35 UV/VIS spectrometer.

2.2.2 Supercritical Fluid Extraction (SFE)

(1) Principle
There are three phases of the matters. They are gas, liquid and solid. As the temperature and the pressure are changing, these phases will interconvert to each other. Besides these phases, actually there are more, for example, plasma and supercritical phases. A supercritical phase is a state in which material can be either liquid or gas, and it appears above the critical temperature and critical pressure where gases and liquids can coexist. Also it shows unique properties that are different from either gases or liquids under standard conditions. A supercritical fluid has both the gaseous property of being able to penetrate anything, and the liquid property of being able to dissolve materials. In addition, it offers the advantage of being able to change
the density into a great extent in a continuous way. On this account, the application of carbon dioxide in the form of a supercritical fluid offers a substitute for an organic solvent used in extraction process in the fields of food industry and medical supplies. SFE is one of the advanced extraction technology where organic solvent is being commonly replaced by supercritical CO2 fluid. In short, it is governed by the principle that solute, the extracted substance, has a very high solubility in CO2 in supercritical state, but has a low solubility in CO2 in normal state. Extraction and separation can be achieved by this principle. SFE of plant secondary metabolites, with pure or modified CO2 preceding an analytical determination, is now recognized as a very powerful technique (Chen and Xue, 2003). SFE machine is consisted of many parts including gas container, pumps, temperature controller, heater, condenser, collector, sample extractor and main controller.

(2) Procedure

SFE instrument (ISCO Model 260D), consisting of one gas container, two pumps, one temperature controller, one heater, one condenser, one collector, one sample extractor and one main controller (Gcyline Ltd. Japan), was used for extraction. Sample was weighted accurately and then put in the extractor of SFE. The temperature in the extractor was adjusted by the temperature controller. The pressurized liquid CO2 was transferred to the extractor by a high pressure pump until the critical pressure of CO2 was reached. Then in the extractor, the CO2 was in a supercritical state. And the extracted substances from the sample had a very high solubility to the supercritical
CO₂, and they dissolved into the supercritical CO₂ fluid. After a few hours, the pressurized CO₂ was released together with the volatile oil into a conical flask with hexane. The extraction process was achieved. And the un-pressurized CO₂ returned to its normal gaseous phase, also the solubility of the extracted components decreased at the same time. The extracted substances were released into the hexane solution. Then the hexane was removed by a rotary evaporator, and the substances extracted by SFE were obtained.

2.2.3 Assay of LIG

2.2.3.1 HPLC Assay of LIG

Instrumentation and Condition

Please refer to 2.2.1.2.

Sample Preparation

1.5mg LIG reference was weighted accurately and put into a 10ml volumetric flask, dissolved into methanol, mixed well and then used as the standard solution. Also the tested samples were weighted accurately and put into a 100ml volumetric flask, dissolved into methanol, mixed well and used as the sample solution.
Calibration Curve

According to the HPLC condition mentioned before, 1, 2, 4, 6, 8 and 10 µl of the standard solution was injected and tested for three times each. The mean peak area was recorded. A graph was plotted with weight as the x-axis and peak area as the y-axis. The regression equation was: \( y = 764.67x + 0.6 (r=0.9997) \). It indicated that the linear relationship of LIG within 0.15-1.5 µg was reliable.

Precision Testing

According to the HPLC condition mentioned before, 10 µl of the standard solution was injected and tested for six times. Each peak area was measured. The RSD was 1.0%, indicating that the accuracy of the HPLC method was good.

Stability Testing

According to the HPLC condition mentioned before, 10 µl of the standard solution was injected for test at 0, 2, 4, 6, 8 and 10 hours. The peak area was measured. And the RSD was 1.8%, indicating that the LIG in methanol was stable for at least 10 hours.

Reproducibility Testing

From the same sample, 6 testing samples were prepared with accurate weight at 0.05 g per each. Under the same conditions of HPLC, for each sample, 10 µl was injected for test. The peak areas were measured and the contents were calculated. The RSD turned
out to be 1.4%, indicating that the reproducibility of this method was good.

**Recovery Testing**

From the same sample, 6 testing samples were prepared with accurate weight at 0.05g per each. Then 0.5, 0.5, 1, 1, 2, 2ml of the LIG standard solution (4.2mg/ml) was added into them respectively. According to the sample preparation, prepared the sample for recovery testing. Under the same HPLC conditions, the recovery rates were calculated. The average recovery rate was 99.2% and the RSD was 1.1%, indicating that the recovery of this method was good.

**Samples Testing**

The HPLC method was carried out. 10µl of the standard and the sample solution was injected into column respectively and examined for 3 times. The areas for the LIG peak were recorded. The standard solution of LIG was prepared afresh daily and was randomly interspersed among the experimental samples during the analysis. The LIG content of the sample was calculated by contrasting the concentration of the standard solution.

**2.2.3.2 UV Assay of LIG**

**Instrumentation and Condition**

Please refer to 2.2.1.5.
**Sample Preparation**

2mg of LIG reference was weighted accurately and placed into a 25ml volumetric flask. Then it was dissolved into methanol and mixed well. 2, 1, 0.5, 0.25, 0.125 as well as 0.0625 ml of the above solution were taken in a series of volumetric flasks respectively, then dissolved into methanol and mixed well to be used as the standard solutions. The tested samples were weighted accurately and put into a 100ml volumetric flask, dissolved into methanol, mixed well and used as the sample solution.

**Calibration Curve**

According to the UV condition mentioned before, methanol was used as the blank solution, and the UV absorbance of LIG in each standard solution was examined for three times, each at 280nm. The mean absorbance was recorded. And a graph was plotted with LIG concentration (C) as the x-axis and UV absorbance (A) as the y-axis. The regression equation was: \( A = 117821C - 0.0035 \) (r=0.9998). It indicated that the linear relationship of LIG within 0.2-6.4µg/ml was reliable.

**Precision Testing**

According to the UV condition mentioned before, the same LIG standard solution was examined for six times. Each absorbance was recorded. The RSD was 0.8 %, indicating that the accuracy of the UV method was good.
Stability Testing

According to the UV condition mentioned before, the same LIG standard solution was examined at 0, 2, 4, 6, 8 and 10 hours respectively. Each absorbance was recorded. The RSD was 2.1%, indicating that the LIG in methanol was stable for at least 10 hours.

Reproducibility Testing

From the same sample, 6 testing samples were prepared with the same accurate weight. In the same condition of UV, for each sample, the absorbance was measured and the content was calculated. The RSD was 2.4%, indicating that the reproducibility of this method was good.

Samples Testing

The UV method was carried out. The standard and sample solutions were examined for 3 times, and the UV absorbance of LIG was recorded. The standard solution of LIG was prepared afresh daily and was randomly interspersed among the experimental samples during the analysis. The LIG content of the sample was calculated by contrasting the concentration of the standard solution.

2.2.4 Isolation Method of Column Chromatography
2.2.4.1 Pressurized Column Chromatography (PCC)

(1) Principle

The column is filled with silica gel as sorbent and the low toxicity solvent, made from petroleum ether and ethyl acetate, is used as eluting solvent. Elution isolation is performed under high pressure offered by the air pressure pump. This is a common method in the isolation experiments.

(2) Procedure

The detailed procedures of PCC were as follows:

**Column Making.** The column was made by the silica gel and petroleum ether, and pressed tightly by air pressure pump.

**Sample Loading.** The sample was mixed up with silica gel adequately, and the mixture was taken to the top of the silica gel column.

**Eluting.** Eluted with the mixture solvent of specific volume ratio of petroleum ether and ethyl acetate, and the eluate was collected with an automatic sample collector.

**Combination.** The eluate was combined with the same compound, and the petroleum ether as well as ethyl acetate of the eluate were removed by a rotary evaporator. After that, the isolated compound was obtained.
2.2.4.2 Decompression Column Chromatography (DCC)

(1) Principle

Silica gel is used as sorbent, while petroleum ether and ethyl acetate are used as the eluting solvent. The DCC isolation is performed with buchuer funnel as the isolation equipment. This method can realize rapid isolation conveniently under low pressure. The isolation condition can be appropriately adjusted each time based on the actual situation, such as changing the diameter of the buchuer funnel, the polarity of the solvent or the amount of the samples collected until the purity of the isolated compound meets the requirement. This is a rapid isolation method. Moreover, it can be stopped anytime without affecting the isolation effect.

(2) Procedure

The detailed procedures were as follows:

**Column Making.** The buchuer funnel was filled with silica gel and the gel was condensed with a pressure reducing pump.

**Sample Loading.** Sample was mixed up with silica gel adequately, and the mixture was put on the top of the buchuer funnel which had been filled with silica gel.

**Elution.** The mixture was eluted with a specific volume ratio of petroleum ether and ethyl acetate, and the eluate was collected sequentially.
Combination. The eluate was combined with the same compound, and the petroleum ether as well as ethyl acetate of the eluate was removed by a rotary evaporator. After that, the isolated compound was obtained.

2.2.5 Determination of Chemical Constant

2.2.5.1 Determination of Boiling Point

Due to its comparatively high boiling point, the compound is maybe unstable at high temperature. Thus, microdose ebulliometry is adopted to measure the boiling point under normal pressure. The boiling point under low pressure is also determined by using vacuum distillation.

(1) Microdose Ebulliometry

Sample was dropped into the outer tunnel of a microdose tube for ebulliometry; nitrogen was filled into the tube to expel oxygen, and the liquid column was about 1 cm in height. The boiling point was measured rapidly in the oil bath at high temperature after the microdose tube for ebulliometry was attached to the thermometer. When heated, small air bubbles in the inner tube escaped slowly due to gas expansion; small air bubbles would escape quickly and consecutively at the boiling point of sample. At this moment the heating could be stopped to decrease the temperature of the oil bath, and the escaping velocity of the air bubbles would
gradually slow down. When the air bubbles failed to burst and were about to retract back to the inner tube, it showed the vapor pressure inside the capillary tube was the same as the ambient pressure. At this moment, the temperature was at the boiling point of sample. For the purpose of calibrating, we heated up the tube slowly after the temperature had decreased by several degrees and when a great deal of air bubbles appeared, the temperature was recorded. The difference between the two readings on thermometer should be less than one degree centigrade.

(2) Vacuum Distillation

The testing facility was similar to the pressure reducing distillation. It was connected to a decompression pump to create vacuum. Sample was taken into a flask of the testing facility, and nitrogen was filled into the facility to expel oxygen. When the vacuum reached a certain level, sample was subjected to flash heat. The boiling point of sample under such vacuum was the temperature at which sample was being steadily distilled. Then the temperature and the vacuum degree were recorded.

2.2.5.2 Determination of Density

A clean and dry picnometer was taken and weighed accurately. After being filled up with sample, a cork was plugged in the picnometer with a capillary located at the center. Any sample overflowing from the taphole was wiped off with filter paper. The picnometer was then placed in a water bath with temperature kept constantly at 30°C
for 30 min. The overload sample would overflow from the taphole continuously, so the top of cork should be wiped with filter paper all the time. At the moment the solution stopped overflowing from the taphole, took the picnometer out of the water bath immediately. The body of the picnometer should be wiped with filter paper and then weighed accurately. The weight of the picnometer was subtracted to reveal the weight of sample. Then the sample was discarded. The picnometer was cleaned and filled with deionized water. The weight of the deionized water at the same temperature was then measured according to the above method. And the density of sample was the ratio of the weight of sample to that of water.

2.2.5.3 Determination of Solubility

An appropriate amount of sample was taken into a stopple-matched standard test tube. Certain quantity of deionized water was added and then the test tube was subjected to sonication for 5 min. After that, it was placed into a swing bed for pendulation (100r/min) at a constant temperature (25°C). The tube was taken out after 24h and underwent centrifugation (3000 r/min) for 15 min. The solution in the underlayer was sucked and then filtered by a micropore filtration film. The filtrate was diluted and the content of sample was determined by relevant method. The determination was carried out according to different gradient concentrations for 3 times repeatedly. The solubility of sample in water was then calculated.
2.2.5.4 Determination of Lipid-water Distribution Coefficient

Fresh phosphate buffer solution at pH 7.4 was added into certain quantity of n-octanol for saturation for 24 h. The buffer phosphate was mixed up with the same volume of n-octanol. Certain quantity of sample was added into the mixture and subjected to sonication for 15 min, and then it was placed in a swing bed at constant temperature (37°C) for pendulation (100r/min), then took out and kept standing until demixing appeared 8 h later. After separation, the sample concentrations in the two phases were determined by relevant method. The lipid-water distribution coefficient was then calculated.

2.2.6 Stability Testing Method

The stability testing can provide information on the quality variation of a drug substance or drug preparation with time under the influence of a variety of environmental factors, such as temperature, humidity and light, to substantiate the recommended manufacture, package, storage, transportation and shipment conditions, and shelf lives of the drug concerned to establish. The basic requirements for stability testing include: factors affecting stability testing, accelerated testing and long-term testing. But long-term testing couldn’t be done in common experiment because of the limited experiment time.
2.2.6.1 Affecting Factors Testing

(1) Principle

This testing is normally carried out under more severe conditions than those used for accelerated tests. The purpose is to investigate the intrinsic stability of the drug substance in order to identify the likely degradation pathways and degradation products, which could provide the rational scientific evidence for manufacturing, package, storage of the drug preparation and establishment of the analytical method for related substances.

(2) Procedure

High Temperature Testing

The drug substance was placed in a suitable clean and tightly closed facility at 60°C for several days. The sample was taken out at different time points and tested for the specified items for stability testing. Where the substance changes significantly, the additional testing at 40°C should be conducted.

High Humidity Testing

The drug substance or drug preparation was placed in a closed facility with constant humidity 90±5% RH for several days at 25°C. The sample was taken out at different time points and tested for the specified items for stability testing. The weight of the substance before and after testing was weighed accurately to evaluate the hygroscopic
properties of the substance. Where more than 5% increase of weight happened, an additional testing with 75% ± 5% RH at 25°C should be conducted. Conditions of constant humidity were made up by placing a saturated salt solution in the lower part of the closed container. According to the requirements for relative humidity, saturated solutions of NaCl (75% ± 1% RH at 15.5°C to 60°C) and KNO₃ (92.5% RH at 25°C) could be used appropriately.

**Photostability Testing by Strong Light**

The drug substance or drug preparation was placed in a light cabinet or other suitable light emitting device. The drug being examined should be exposed to strong light for several days. The sample was taken out at different time points and tested for the specified items for stability testing. Any changes in appearance of the substance should be noticed.

According to the chemical, physical and microbiological properties of the drug, experiment could be designed when necessary for exploring the affect of pH, oxygen and other factors on stability of the drug. For innovate or new drugs, it is necessary to study the properties of their decomposition products.

**2.2.6.2 Accelerated Testing**

**(1) Principle**
This testing is carried out under exaggerated storage conditions. The purpose is to predict stability of the drug preparation by accelerating the chemical or physical change of drug preparation and provide the necessary date for evaluation, manufacturing, transportation and storage of the new drug.

(2) Procedure

The drug preparation should be stored under the conditions of accelerated testing at high temperature, high humidity and strong light for several days. The preparation should be examined respectively at different testing periods according to the specific items for stability testing.

2.2.7 Synthesis of LIG

2.2.7.1 Synthesis of 3-Hydroxy-3-butylphthalide

Without any water and oxygen, phthalic anhydride was used as raw material while anhydrous THF as solvent. Then butyl lithium was added to react, and butyl from butyl lithium was adopted as introductive functional group. After acidifying, extracting and desiccating, 3-Hydroxy-3-butylphthalide was obtained.

2.2.7.2 Synthesis of 3-butylidene-phthalide
3-Hydroxy-3-butylphthalide was dissolved into Benzene, and a little amount of P-T$_3$OH was added into it. The mixture was stirred with heating. Then it was dried by anhydrous sodium sulfate after the mixture was washed by saturated NaCl solution. Finally 3-butylidene-phthalide was obtained after the solvent was removed through decompression.

### 2.2.7.3 Synthesis of LIG

3-butylidene-phthalide was dissolved into anhydrous THF. After that, Lithium, 2-butyl alcohol as well as fluid ammonia were added into at -60°C. Then the mixture was stirred adequately. During the stirring period, the mixture turned blue gradually. Ammonium chloride was added until the blue colour faded away. Then the mixture was melted by ice and acidified by HCl till pH between 5 and 6. And then it was extracted by ether and dried by anhydrous sodium sulfate. At last, LIG was obtained after the mixture was isolated by silica gel column chromatography method.

### 2.2.8 Preparation Process of LIG CD Inclusion

**(1) Principle**

Drug molecules can be included in CD in 2 major forms, which are 1:1 or 1:2 as guest (drug) to host (CD) molecule ratio. The formation of inclusion complexes, which is a
physical process, does not involve any chemical reaction or formation of ionic and covalent bond (Yoshii et al., 2006). The formation of inclusion complexes depends on the polarity of the host and guest molecules. There are different methods could be used to prepare inclusion complexes.

(2) Procedure

HP-β-CD was chosen as the host substance of LIG CD inclusion complexes in this experiment. It was taken and dissolved into deionized water at 50°C in a conical flask with a magnetic bar providing a stirring force to enhance the dissolution of HP-β-CD, thus ensure all HP-β-CD was dissolved. Then the solution was allowed to cool down to room temperature. Then LIG was added to prepare the HP-β-CD solution. In order to determine what method could give the best efficiency, we had tried different methods to prepare LIG CD inclusion complexes. Besides, the formation of inclusion complexes was affected by extrinsic conditions such as reaction time, reaction temperature, the time for stirring (or ultrasonic concussion), and the concentration of the reactant. Generally, the formation of inclusion complexes was in favor of a longer reaction time and stirring time, as well as an enhanced concentration of CD. The single factor tests were done to optimize the best preparation process by comparing the efficiency of different preparations on the LIG-CD inclusion complexes using the physical stability of LIG-CD solutions as the index.

2.2.9 Preparation Process of LIG ME
(1) Principle

Micro-emulsion is a mixture composed of multi-components, with at least three, usually four or five components. Single, double or triple phase can be obtained by changing the parameters of the system. Phase diagram is the basic tool for the study of micro-emulsion. The determination of the phase border is very important in the study of micro-emulsion. The formulation design of ME relies on the proper selection and ration of components to enable the fitness of the components for pharmaceutical purpose and ME formation in a wide range.

(2) Procedure

In the preparation of micro-emulsion, emulsifier, co-emulsifier and oil phase were mixed well, and LIG was added into. Then the mixture was mixed up till it became a transparent yellow fluid. After that, deionized water was slowly added into the mixture, mixed thoroughly and observed immediately. Single factor tests were adopted to determine the best emulsifier, the best oil phase and the ratio of emulsifier phase to oil phase. Pseudo-ternary phase diagram was plotted by taking an emulsifier and a co–emulsifier as the emulsifier phase, vegetable oil and LIG as the oil phase, and deionized water as the aqueous phase. At 40°C, the emulsifier phase, oil phase, and aqueous phase in different volume were taken into a beaker and mixed thoroughly, then the phenomenon change was observed with the color transferred from clear to thick and then back to clear. The percentage of all components at critical points was
recorded. Then pseudo-ternary phase diagram was drawn with the percentage of all components at critical points. The optimum LIG ME formulation would be obtained at the O/W micro-emulsion zone in the pseudo-ternary phase diagram.

### 2.2.10 Acute Toxicity Test

(1) Principle

The objectives of acute toxicity test are to define the intrinsic toxicity of the chemical, identify the target organs, and predict the hazard to non-target species. This can not only provide information for risk evaluation of acute exposure to the testing chemical, but also provide information for the design and selection of future studies of dose level. Most importantly, it also provides information for clinicians about acute over-exposure or poisoning. Acute toxicity studies are the first defending line before long-term studies (Davis, 2002). LD$_{50}$ is the value which is commonly used in acute toxicity studies. And it is also an index to define acute toxicity. The acute toxicity test under different conditions and exposure routes is considered. NIH mice are used usually in the acute toxicity test to find out the LD$_{50}$ using a variety of dosages by intragastrical and intraperitoneal injection (ig and ip).

(2) Procedure

The test substance was given in diluted form at different dosages. It was diluted with appropriate solvent such as 3% Tween-80. Dosages (from 200mg/kg to 1400mg/kg)
were generally based on the body weight of the animals. The symptoms of the animals after injection and the time of death were recorded. The observational index included appearance, activity, psychosis, urinary and fecal discharges, breath, secretion of nasal, eyes and mouth, weight as well as death. Dead mice should be dissected and have pathological organs check. Observation was done for 2 weeks and animals were weighted and recorded at a 7-day interval. If animals survived throughout the two weeks, they were killed by breaking their backbone. Then, they were dissected to see if there were any abnormalities. If yes, abnormality was recorded. The mortality rate of different dosage groups was calculated. Then, the software LD50 was used, with the method of Bliss to find out the value of LD50 in the two ig and ip tests (Gad and Chengelis, 1998).
CHAPTER 3

VOLATILE OIL EXTRACTION FROM ANGELICA SINENSIS

3.1 ABSTRACT

LIG is the active constituent of Angelica sinensis (当归) and Ligusticum chuanxiong (川芎). The content of LIG in Angelica sinensis and Ligusticum chuanxiong was compared by the methods of TLC, HPLC, GC and GC-MS in this study, and it was found that there was a higher content of LIG and less other similar compound in Angelica sinensis. So Angelica sinensis was the ideal raw material to extract LIG. The extraction process for Angelica sinensis volatile oil (ASVO) was investigated with comparisons of steam distillation, ethanol extraction and supercritical fluid extraction (SFE). The results showed the SFE was the best method for extracting ASVO. The uniform design was further used to optimize the extraction process of SFE. The optimum conditions for SFE were: 50°C, 5500Ps, 10% ethanol modifier and 4 hrs of extracting time.

3.2 INTRODUCTION

The content of LIG was high in Angelica sinensis and Ligusticum chuanxiong, two
kinds of Traditional Chinese Medicine (TCM). It could reach up to almost 1% in *Angelica sinensis* and *Ligusticum chuanxiong* from the Good Agricultural Practice (GAP) cultivating base. Moreover, the output of both *Angelica sinensis* and *Ligusticum chuanxiong* was quite high to meet the demand of the industrial production of LIG. However, the synthesis of LIG was difficult due to its specific structure. Even though there were a few papers (Li et al., 1994; Ogawa et al., 1995) which had mentioned the synthesis of LIG, saying its low yield made its industrial production difficult. As a result, up to now, LIG has mostly been isolated from *Angelica sinensis* and *Ligusticum chuanxiong*. There have been a few of investigations that focused on what kind of material was more suitable to isolate LIG. In order to find out the best material for LIG production and bring convenience to the isolation research for industrial production, TLC, HPLC, GC and GC-MS methods were performed using the reference of LIG as the control.

Since LIG is a component of the volatile oil, the first isolation step of LIG begins from the extraction of the volatile oil. There are several commonly used methods in modern industry (Fang et al., 1979; He et al., 1996; Xiao et al., 2002), such as steam distillation, organic solvent extraction and SFE, and each of them has its own advantages and disadvantages.

Steam distillation is the most convenient and common method, which uses vapour to
extract components. The advantage of this method is its low cost. However, the high temperature during extraction could destroy thermolabile components. Since LIG is thermolabile, the content of LIG extracted by this method would be low, but this method is still often used in industry due to its convenience (Li et al., 1995).

Organic solvent extraction is usually used to extract low polar components and volatile oil, which could dissolve in organic solvent (ethanol or petroleum ether), and could be extracted through decoction, infiltration, immersion and backflow steps. Ethanol is one of the most common organic solvents due to its low price and low toxicity. So ethanol extraction is a typical method of organic solvent extraction. It is the most commonly used method in industrial extraction because of its easy manipulation and high extraction rate. Its disadvantage is that a large amount of high polar materials such as sugar is often extracted when ethanol is used as the solvent. Complex procedures are therefore required to purify the extracted volatile oil (Bai et al., 2003)

SFE is a new technique for extraction and isolation (Li et al., 1996). As fluid solvent in the supercritical area near the critical point possesses the abnormal phase balance behavior and transmission capability, the components in the material could be isolated by the fluid solvent within a wide range base on the changing of pressure and temperature. SFE is one of the advanced extraction technologies where CO2 is the common supercritical fluid. The extracted compounds have a very high solubility in
CO2 in supercritical state, but they have low solubility in CO2 in normal state. Theoretically, the volatile oil could be extracted. The SFE method with extraction and distillation features could be used in low temperature with no remaining solvent, and it only requires a short operation time with high efficiency, also be applicable for extraction of thermosensitive components and could prevent them form oxygenating and degrading. Despite these advantages, its expensive equipment has limited its application. As the high demand for quality of the extracted composition, SFE was largely used in the extraction application of the products with high quality, high purity and low impurity (Huang et al., 2002).

Scientific experiments are very important for our studies and are commonly performed in our lab. However, when the experiments involve many factors, things seem to be more difficult to carry out. For example there are 5 factors to one experiment, and each factors have 10 levels, it need to perform $5^{10} = 9765625$ experiments. Since time always means money, too many experiments are not economically acceptable. It led to the development of some statistically designed tools to perform the experiments. Professor Fang KT and Professor Wang Y proposed one of famous experimental designs in 1980, Uniform Design, which had been applied in many aspects successfully, for example, natural sciences, chemical engineering, pharmaceutics, surveying design and computer sciences (Liu, 2002). In this design, the experiment, with 5 factors and 10 levels for each factor, could be completed in 10 times instead of 9765625. It had greatly saved the time and money. Also the result
was significantly reliable. So here in our study, in order to optimize the extraction process of the volatile oil in *Angelica sinensis*, uniform design would be used to set the relative conditions.

By using the optimized medicine material which contains high content of LIG, and regarding the industrial conditions, experiments were conducted to compare these 3 different extraction methods for volatile oil in order to find out the best to extract volatile oil containing LIG and then further optimize the extraction process with uniform design.

### 3.3 MATERIALS AND METHODS

#### 3.3.1 Materials

**3.3.1.1 Materials**

HPLC grade methanol was purchased from Honeywell International Inc., Muskegon, USA and isopropyl alcohol was from Tedia Company, Fairfield, USA. Radix *Angelica sinensis*, known as Danggui in Chinese, was purchased from the GAP cultivating base in Min Xian County, Gansu Province, China. Rhizoma *Ligusticum chuanxiong* was purchased from the GAP cultivating base in Guan County, Sichuan Province, China. Their identity was confirmed by comparison to descriptions of
characteristics and appropriate monograph in the Chinese Pharmacopoeia (The State Pharmacopoeia Commission of People’s Republic of China, 2000). Liquid CO₂ was purchased from Chun Wang Industrial Gas Ltd. Shenzhen, China. 95% ethanol was from Dai Mo Chemical Co, Tianjin, China. Silica gel GF₂₅₄ plate was from Ocean Chemical Ltd. Qingdao, China.

3.3.1.2 LIG Preparation

LIG was extracted, separated and purified from Radix *Angelica sinensis* in our laboratory, with batch number: 050118. Purified liustilide was identified by electron impact ionization (EI) MS, H¹ NMR and C¹³ NMR spectrometric techniques. The purity was found to be > 98% based on the percentage of total peak area by GC analysis.

3.3.2 Methods

3.3.2.1 Components Assay of *Angelica sinensis* and *Ligusticum chuanxiong*

3.3.2.1.1 Preparation of Solution

3.3.2.1.1.1 Preparation of Standard Solution
1.5mg of LIG reference was weighted accurately and put in a 10ml volumetric flask, dissolved and diluted with methanol to volume, mixed well and used as the standard solution.

3.3.2.1.2 Preparation of Test Solution

3 batches of Radix *Angelica sinensis* and Rhizoma *Ligusticum chuanxiong* were mixed with same weight and smash to pass though No.3 sieve, respectively. Then 10g of *Angelica sinensis* and Rhizoma *Ligusticum chuanxiong* powder were weighed accurately, after that 100ml, 80ml, 60ml of methanol were added to extract 3 times for 30min with ultrasonic instrument respectively, take out, add methanol to 250ml, mix well, filter and use as the test solution of *Angelica sinensis* and *Ligusticum chuanxiong*, respectively.

3.3.2.1.2 TLC Assay

Please refer to 2.2.1.1.

3.3.2.1.3 HPLC Assay

Please refer to 2.2.1.2.
3.3.2.1.4 GC Assay

Please refer to 2.2.1.3.

3.3.2.1.5 GC-MS Assay

Please refer to 2.2.1.4.

3.3.2.2 Volatile Oil Extraction Methods Contrast

3.3.2.2.1 Steam Distillation

Steam distillation was a traditional method to extract the *Angelica sinensis* volatile oil (ASVO). In order to compare the yield and the purification of the LIGs, this method was performed. 100g Radix *Angelica sinensis* was weighed accurately in a 2000 ml flask of volatile oil extractor. 1000ml of water was added and heated with an electronic heater jacket. The oil with water was collected to a beaker at different time intervals: 0-0.5h, 0.5-1.0h, 1.0-2h, 2-3h, 3-4h, 4-6h, 6-8h, 8-10h, 10-14h. Ether was added to extract the oil from the oil water mixture, and then anhydrous Na$_2$SO$_4$ was added to dry the remaining water in the ether. The ether was removed by a rotary evaporator, and the volatile oil extracted by steam distillation was obtained. The
volume of the volatile oil was examined by graduated pipette, and the LIG content of the volatile was assayed by HPLC.

3.3.2.2.2 Ethanol Extraction

Ethanol extraction was a type of organic solvent extraction and another common extraction method of the volatile oil. It was also chosen to compare with the extraction method of ASVO. 100g Radix *Angelica sinensis* was weighted accurately and put in a 2000 ml flask. 800ml, 600ml, 400ml of 95% ethanol was added to boil extract 3 times for 1 hour with the electronic heater jacket respectively. Taken out, stored the ethanol mixture in 4 °C for 24 hour in order to precipitate the sugar. Then filtered the mixture and concentrated with a rotary evaporator, and the volatile oil extracted by ethanol distillation was obtained. The volume of the volatile oil was examined by graduated pipette, and the LIG content of the volatile was assayed by HPLC.

3.3.2.2.3 Supercritical Fluid Extraction (SFE)

SFE instrument (ISCO Model 260D), consisting of one gas container, two pumps, one temperature controller, one heater, one condenser, one collector, one sample extractor and one main controller (Gcyline Ltd. Japan), was used for extraction. 100g Radix
*Angelica sinensis* was weighted accurately and put in the extractor of SFE. The temperature in the extractor was adjusted by the temperature controller. The pressurized liquid CO\(_2\) was transferred to the extractor by a high pressure pump until the critical pressure of CO\(_2\) was reached. In the extractor, the CO\(_2\) was in a supercritical state. The extracted substances, *Angelica sinensis* volatile oil (ASVO), had a very high solubility to the supercritical CO\(_2\), and they dissolved into the supercritical CO\(_2\) fluid. After a few hours, the pressurized CO\(_2\) was released together with the volatile oil into a conical flask with hexane. The extraction process was achieved. The un-pressurized CO\(_2\) returned to its normal gaseous phase and the solubility of the extracted components decreased at the same time. The extracted substances were released into the hexane solution. Then the hexane was removed by a rotary evaporator, and the volatile oil extracted by SFE was obtained. The volume of the volatile oil was examined by graduated pipette, and the LIG content of the volatile was assayed by HPLC.

### 3.3.2.4 HPLC Assay of LIG

Please refer to 2.2.3.1.

### 3.3.2.3 Optimized SFE Process with Uniform Design
There were some reports about the optimization extraction condition for the ASVO (Hu and Ding, 2003). However, most of them were concerned on the extraction yield only. In this study, the quality of LIG was also taken into consideration. The results of the different extractions showed that the SFE was the best extraction method for ASVO. The extraction process of SFE was complex and affected by many factors. The most important things were the nature of the extracted solutes and extracting solvents. But they were affected by many physical factors, for example, pressure, temperature, extraction time and the selection of modifiers (S. Hawthorne, 1993). They were analyzed in the following.

**Pressure**

Pressure is one of the most important parameters in the SFE process. Temperature being constant, an increase in pressure will lead to an increase in fluid density. The extraction effect will be easily changed by the pressure, which is around the critical pressure point. Different substances have different extraction pressure. Generally speaking, the pressure for extracting substances with -OH, -COOH is higher than that for hydrocarbons and esters. The maximum pressure provide by the machine is 7500 Psi, so the range of pressure being tested was from 2000 to 7000 Psi.

**Temperature**

Temperature is another important factor governing the extraction process. It has both positive and negative effects on the solubility of solute to the supercritical fluid. On
the negative side, as temperature increases, the density of the supercritical fluid decreases. This leads to the decrease of the solubility of solute to the supercritical fluid. As a result, the extraction yield becomes lower. In the positive side, increase in temperature makes the solutes more volatile. The concentration of volatile solute in the supercritical fluid increases. As a result, the extraction yield becomes higher. The net effect of temperature on SFE could be found only via experiments. Also, higher temperature could destroy or inactivate the extracted substances. The tested temperature range was from 30-110°C.

**Selection of Modifier**

CO₂ is the most commonly used extraction solvent of SFE due to its many advantages: it is safe, environmental friendly, cheap and non-flammable. Nitrogen and methane could be other alternatives. However, they have a common feature to SFE. They are slightly polarized. This property is suitable to extract hydrophobic substances. But for hydrophilic substances, the solubility of them to supercritical fluid was low. As a result, the extraction yield decreased. In order to solve this problem, a modifier was needed. By mixing a polar solvent into the CO₂, the nature of fluid solvent changed to slightly polarity and improved the extraction yield of the polar substance. So, before the addition of the modifiers, it was a must to know the properties of the substances being extracted. Ethanol absolute (99.7%) was chosen to be the modifier in this study. The percentage range of the modifier (ethanol absolute) was from 0 to 35%.

**Extraction Time**
Generally speaking, the extraction yield increases with the extraction time. If enough time is offered, the substances being extracted could mostly dissolve into the supercritical fluid. However, a longer duration means an increase of cost of the whole process. This included the costs of CO$_2$, modifiers and electricity. In this study, the extraction time ranged from 45mins to 6 hours.

These four factors on the extraction of ASVO were investigated in this study to find the optimum condition using the uniform design. In this study, there were 4 factors and nine levels. So the following Table3-1 and 3-2 of the uniform design were chosen to optimize the extraction process of SFE. According to the conditions of the uniform design, the ASVO of each uniform design condition was extracted by using SFE for 3 times. Then the volatile oil was obtained, and the LIG content of the volatile oil was examined by the above-mentioned HPLC method.

3.4 RESULTS

3.4.1 Components Assay of *Angelica sinensis* and *Ligusticum chuanxiong*

3.4.1.1 TLC Assay (Fig3-1)

The TLC method was carried out, the LIG standard solution and the test solution of
Angelica sinensis and Ligusticum chuanxiong were taken in the silica gel plate to develop, removed the plate, dried in the air, examined under ultraviolet light (254nm and 365nm) and dyed with iodine vapor, then the chromatogram was recorded (Fig 3-1). From Fig3-1, it showed that there was a high LIG content in Angelica sinensis and Ligusticum chuanxiong and other similar polar components.

3.4.1.2 HPLC Assay (Fig 3-2, 3-3, 3-4)

HPLC method was carried out, 10ul of the LIG standard solution and the test solution of Angelica sinensis and Ligusticum chuanxiong was injected, determined and recorded the chromatogram respectively (Fig 3-2,3-3,3-4). The content of LIG was calculated by contrasting the concentration of the LIG standard solution. From Fig 3-2,3-3,3-4, the retention time of liustilide was 9.8 minute. The LIG content was 0.645% and 0.608% in Angelica sinensis and Ligusticum chuanxiong respectively. The LIG content in Angelica sinensis was slightly higher. In the Ligusticum chuanxiong, there were a lot of other similar polar components and the component with retention time 6.2 minute had very high content. The preparation for LIG was relatively difficult if there had many other similar polar components. This result was obvious that Angelica sinensis was the best material for LIG extraction.

3.4.1.3 GC Assay (Fig 3-5, 3-6, 3-7)
GC method was carried out, 0.5ul of the LIG standard solution and the test solution of *Angelica sinensis* and *Ligusticum chuanxiong* was injected and examined, the chromatogram was recorded (Fig3-5,3-6,3-7). From Fig 3-5,3-6,3-7, the retention time of LIG was about 12.5min, and there contained a great deal of LIG in both *Angelica sinensis* and *Ligusticum chuanxiong*. However, *Ligusticum chuanxiong* had also a high content component with retention time about 12.2 min, while *Angelica sinensis* did not. Meanwhile, the peak area normalization analysis showed that the sample of *Angelica sinensis* contained about 45% of liustilide, and that *Ligusticum chuanxiong* contained only about 30%. It was obvious that *Angelica sinensis* was the best material for LIG extraction.

3.4.1.4 GC-MS Assay (from Fig 3-8 to Fig 3-12)

GC-MS method was carried out , 0.5ul of the LIG standard solution and the test solution of *Angelica sinensis* and *Ligusticum chuanxiong* was injected and examined, then the main peak of total ion chromatogram and the mass spectrum was recorded (Fig 3-8,3-9,3-10). From Fig 3-8,3-9,3-10, the retention time of LIG was about 8.4min, and there contained a great deal of LIG in both *Angelica sinensis* and *Ligusticum chuanxiong*. However, *Ligusticum chuanxiong* also had a high content component and the retention time was about 12.2 min, while *Angelica sinensis* did not. The component was senkyunolide because the mass spectrum (Fig 3-11) was the same as the structure of senkyunolide in the published report (Luo et al., 1996). As the
structure of senkyunolide was similar to the structure of ligustildie (Fig 3-12), senkyunolide would be the main interferential compound for LIG isolation. From this result, it was obvious that *Angelica sinensis* was the best material for LIG extraction.

### 3.4.2 Volatile Oil Extraction Methods Contrast

#### 3.4.2.1 Volatile Oil Volume Contrast (Table 3-3)

According to 3 different extraction methods, 100g Radix *Angelica sinensis* was weighed accurately and extracted the volatile oil using steam distillation, ethanol extraction and SFE respectively. The volatile oil was obtained, and the oil volume was examined by graduated pipette. The result was shown in the Table 3-3.

#### 3.4.2.2 LIG Assay Contrast

HPLC method was carried out, 10ul of LIG standard solution and the different test solution was injected, determined and recorded the chromatogram respectively. The content was calculated by contrasting the concentration of the LIG standard solution. The result was in Table 3-3.

From Table 3-3, the smallest amount of volatile oil and LIG was obtained by steam distillation, as volatile oil could not been completely extracted and a great part of the
extracted ligstilide had been destroyed by high temperature. The largest amount of volatile oil and LIG was obtained by ethanol extraction, but the concentration of LIG was very low, which made the subsequent isolation of LIG very difficult. In comparison, a moderate amount of volatile oil and a relatively high amount of LIG were obtained by SFE, and the concentration of LIG was very high. Thus SFE was the most suitable method for the extraction of the ASVO.

3.4.3 Optimized SFE Process with Uniform Design (Table 3-4,3-5)

According to the conditions of uniform design, the ASVO of each uniform design condition was extracted by using SFE for 3 times. Then the volatile oil of each uniform design condition was obtained, and the LIG content of the volatile oil was examined by the above-mentioned HPLC method. The optimization of SFE factors was based on the regression equation and related data from HPLC. The amount of LIG was chosen to be the index. The result was shown in the Tables 3-4,3-5.

As shown in Tables 3-4,3-5, we had applied the software of the uniform design to estimate the optimum conditions for SFE of *Angelica sinensis*.

By using the software, the equation was:

\[ Y=0.0101-0.000322A+0.000049B+0.00597C+0.0141D \]

The result:

\[ Y = b(0) + b(1)X(1) + b(2)X(2) + b(3)X(3) + b(4)X(4) \]
Regression coefficient $b(i)$:

$b(0) = 1.01 \times 10^{-2}$

$b(1) = -3.22 \times 10^{-4}$

$b(2) = 4.90 \times 10^{-5}$

$b(3) = 5.97 \times 10^{-3}$

$b(4) = 1.41 \times 10^{-2}$

When the value of regression coefficient was positive, the higher value of factors would be chosen. When the value of regression coefficient was negative, the lower value of the factors would be chosen.

For A temperature, the regression coefficient was $-3.22 \times 10^{-4}$. Since it was negative, a lower temperature $50^\circ C$ was chosen. It was because too high a temperature would damage the active components of the ASVO while too low a temperature would decrease the rate of extraction. (Zhao et al, 2004). The optimum temperature was be $50^\circ C$.

For B pressure, the regression coefficient was $4.90 \times 10^{-5}$. Since it was positive, a higher pressure should be chosen for SFE of *Angelica sinensis*. However, because the maximum pressure of the SFE machine was 7000 Psi, it was better to choose 5500 Psi as the optimum pressure in order to prolong the life of the SFE machine and maintain the safety of the environment of the working conditions.

For C concentration of the modifier, the regression coefficient was $5.97 \times 10^{-3}$. Since it was positive, in order to have the maximum extraction yield, a higher percentage of
ethanol should be used. However, a higher concentration of ethanol would increase
the amount of the oil, but not the concentration of LIG. So, 10% ethanol was chosen
as the optimum of the modifier.
For D extraction time, the regression coefficient was 1.41e^{-2}. Since it was positive, a
longer extraction time should be used. However, a longer duration of each round of
extraction would decrease the turn over rate of the production plant. In other words,
the extraction yield increases with the extraction time. So 4-hour was chosen to be the
optimum time for ASVO extraction.
The optimum conditions for SFE of the ASVO extraction were: 50℃, 5500Psi, 10%
etanol modifier and a 4-hour extraction time.

3.5 DISCUSSION

As it was reported that Angelica sinensis and Ligusticum chuanxiong contained the
high content of LIG, in present study, 3 batches of Angelica sinensis and Ligusticum
chuanxiong were mixed up with equal amount, and then TLC, HPLC, GC and
GC-MS were performed by using the mixture as well as the LIG reference which was
used as the control for comparison systematically. The results showed that Angelica
sinensis contained higher content of LIG and less impurity with the similar property,
while Ligusticum chuanxiong contained a comparatively lower content of LIG and
more impurity. As a result, Angelica sinensis was the most suitable material to extract
LIG. Moreover, since there are GAP bases of Angelica sinensis in China, Angelica
*sinensis* could have adequate supply to meet the industrial production of LIG.

Steam distillation, organic solvent extraction and SFE are very common in extracting volatile oil and low polar components. LIG is not only a low polar component, but also a component of volatile oil. Therefore, this study contrastively showed the extraction effects of these three methods on the ASVO. The SFE method with extraction and distillation features could be used in a low temperature without any remaining solvent, and it requires a short operation time with high efficiency, also be applicable for the extraction of thermosensitive components and could prevent from oxygenating and degrading. The final results also showed that the SFE is the best method to extract the ASVO, and that was reported by many papers. Therefore, this method is also proven to be very important and have high potential effect to the extraction of TCM.

SFE had been chosen as the optimized method of the ASVO extraction, and then uniform Design was performed to investigate the volatile oil extraction process, in which pressure, temperature, extraction time and the selection of modifiers were chosen as the optimized factors. A better extraction process was obtained after using the uniform design to inspect the whole extraction procedure. Then a basic foundation was laid for the industrial production of the ASVO by SFE.
## Table 3-1. Uniform design table

<table>
<thead>
<tr>
<th>No.</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
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<tbody>
<tr>
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<td>1</td>
<td>2</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
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<tr>
<td>9</td>
<td>9</td>
<td>9</td>
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<td>9</td>
</tr>
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</table>

## Table 3-2. Experiment table based on uniform design

<table>
<thead>
<tr>
<th>No.</th>
<th>Temperature (°C)</th>
<th>Pressure (Psi)</th>
<th>Ethanol (%)</th>
<th>Time (h)</th>
</tr>
</thead>
<tbody>
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<td>3000</td>
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<td>2</td>
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</tr>
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<td>2</td>
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<td>20</td>
<td>1.5</td>
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</tr>
<tr>
<td>9</td>
<td>110</td>
<td>7000</td>
<td>35</td>
<td>7</td>
</tr>
</tbody>
</table>
Figure 3-1. The TLC chromatogram (left: ultraviolet light 254nm, middle: ultraviolet light 365nm, right: Dyed with iodine vapor) The spots of every picture were the LIG reference, the sample of *Angelica sinensis* and the sample of *Ligusticum chuanxiong* form left to right respectively. The Rf of liustilide reference spot was about 0.7.

Figure 3-2. The HPLC chromatogram of LIG reference.
Figure 3-3. The HPLC chromatogram of *Angelica sinensis*.

Figure 3-4. The HPLC chromatogram of *Ligusticum chuanxiong*.
Figure 3-5. The GC chromatogram of LIG reference.

Figure 3-6. The GC chromatogram of Angelica sinensis.
Figure 3-7. The GC chromatogram of *Ligusticum chuanxiong*.

Figure 3-8. The total ion chromatogram of LIG reference in GC-MS.
**Figure 3-9.** The total ion chromatogram of *Angelica sinensis* in GC-MS

**Figure 3-10.** The total ion chromatogram of *Ligusticum chuanxiong* in GC-MS
Figure 3-11. The mass spectrum of the component in *Ligusticum chuanxiong* which its retention time was 8.18 minute in GC-MS. It was proved as senkyunolide.

Figure 3-12. The mass spectrum of LIG in GC-MS.
Table 3-3. The extraction result of ASVO with three different extraction method.

<table>
<thead>
<tr>
<th>Methods</th>
<th>Oil volume(ml)</th>
<th>Lig content (%)</th>
<th>Lig amount(g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steam distillation</td>
<td>0.23</td>
<td>58.529</td>
<td>0.135</td>
</tr>
<tr>
<td>Ethanol extraction</td>
<td>2.0</td>
<td>26.800</td>
<td>0.536</td>
</tr>
<tr>
<td>SFE</td>
<td>0.98</td>
<td>46.444</td>
<td>0.455</td>
</tr>
</tbody>
</table>

Table 3-4. The extraction result of the uniform design of ASVO with SFE method.

<table>
<thead>
<tr>
<th>No.</th>
<th>A (°C)</th>
<th>B (Psi)</th>
<th>C (%)</th>
<th>D (h)</th>
<th>ASVO (g)</th>
<th>LIG(g)</th>
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</thead>
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<td>0.701</td>
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</tr>
<tr>
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<td>6500</td>
<td>25</td>
<td>3</td>
<td>1.210</td>
<td>0.504</td>
</tr>
<tr>
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<td>70</td>
<td>2000</td>
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<td>0.216</td>
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<tr>
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<td>0.818</td>
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<tr>
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<td>0.684</td>
<td>0.345</td>
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<tr>
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<td>7000</td>
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<td>7</td>
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<td>0.630</td>
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</table>

Table 3-5. Variance analysis of the uniform design

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of square</th>
<th>Degree of freedom</th>
<th>Mean square</th>
<th>F-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regression</td>
<td>U=0.175</td>
<td>K=4</td>
<td>U/K=4.39e^{-2}</td>
<td>F=14.85</td>
</tr>
<tr>
<td>Residue</td>
<td>Q=1.18e^{-2}</td>
<td>N-1-K=4</td>
<td>Q/(N-1-K)=2.95e^{-3}</td>
<td></td>
</tr>
<tr>
<td>Sum</td>
<td>L=0.187</td>
<td>N-1=8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

N=9, Coefficient of multiple correlation $R=0.9679$, level of significance $\alpha=0.05$, $F_t=14.85$, $F(0.05,4,4)=6.388$, The standard deviation of residual $s=5.43e^2$. Since $F_t>F(0.05,4,4)$, the regression equation is significant.
CHAPTER 4

LIGUSTILIDE ISOLATION FROM *ANGELICA SINENSIS* VOLATILE OIL

4.1 ABSTRACT

In this study, by using *Angelica sinensis* volatile oil (ASVO) as the material, the effects of pressure reducing distillation (PRD), pressurized column chromatography (PCC) and decompression column chromatography (DCC) were compared for the isolation of ligustilide (LIG). The results showed that DCC was the best method to isolate LIG. This process was subsequently optimized and a new isolation method was established. The pilot plant test of DCC was accomplished to adapt industrial production. The purity of the isolated LIG was examined by TLC, HPLC and GC; the structure of the isolated LIG was examined by CC-MS, NMR, IR and UV. It showed that the isolated component was LIG and the purity of it was higher than 98%.

4.2 INTRODUCTION

Column chromatography is a commonly used method to isolate organic compounds, and silica gel is a common chromatography sorbent. Gravity silica gel column
chromatography has an advantage of convenience, but it also has limitation, such as low isolation efficiency, long duration, etc. As the chromatography technique develops, these special column chromatography, such as PCC, DCC and vacuum liquid chromatography (VLC) are developed accordingly. All of these methods could overcome the shortcomings while preserving the advantages of the conventional column chromatography. As a result, they are widely used in the compound isolation.

In view of the property of LIG, column chromatography was the most suitable method for extraction by using silica gel as the sorbent. Silica gel is the most economic and commonly used sorbent in industrial isolation. As LIG has low polarity, two conventional solvents, petroleum ether and ethyl acetate with low toxicity and low polarity, were needed in isolation by column chromatography.

PCC isolated compounds rapidly through pressurizing the silica gel column by using a pressure pump during elution. The chromatographic column was filled with wet silica gel. Capillary effect was provided from the gaps among the silica gel particles, it could increase isolation efficiency and purity of sample isolation. At present, PCC is widely used in preparative isolation of Chinese herbal components, antibiotics and organic components (Li et al., 2006).

The development of DCC is based on PCC and VLC. It works through reducing the bottom pressure of the chromatography column, so thus to induce the eluate flow out
and realize the isolation rapidly. DCC has advantages such as simple manipulation, quick preparation, simple equipments, short process and high yield. Also it requires less solvent and is independent to time. Moreover, the isolation can be broken off at any time.

Liquid compounds could be isolated at different boiling point. The boiling point is the temperature when the vapor pressure of a liquid equals the atmospheric pressure, thus the temperature of the boiling liquid reduces with the external pressure. Reducing the pressure on the liquid surface by using a vacuum pump could reduce the boiling temperature of the liquid. Distillation under such a condition is called PRD. It is commonly used in isolating and purifying liquid compounds with a high boiling point, unstable property and solid organics with a low fusion point. The compound, which has a high boiling point and is easy to decompose, oxidize or polymerize below boiling point temperature, could be isolated by this method. LIG could be isolated by the PRD method, however, there are many compounds owning similar boiling point with LIG in ASVO which might disturb the isolation.

At present, there are many papers reporting the isolation of LIG, but none of them went beyond the lab level. The isolated LIG was limited to the magnitude of microgram scale, while the grams scale is needed for LIG which was supposed to be developed as medicine for industrial production. This is also one of the key points of this study. PRD, PCC and DCC would be compared to find out a more suitable
isolation method for industrial production according to the property of LIG, and pilot plant test would be carried out subsequently. TLC, HPLC, GC-MS and NMR methods were performed to assess the purity and the structure of the isolated LIG.

4.3 MATERIALS AND METHODS

4.3.1 Materials

HPLC grade methanol was purchased from Honeywell International Inc., Muskegon, USA and isopropyl alcohol was from Tedia Company, Fairfield, USA. Angelica sinensis volatile oil (ASVO) was purchased from Meicheng Pharmacy Ltd., Guangzhou, China. Ethyl acetate was from Beijing Chemical Co., Beijing, China. Methanol, hexane and petroleum ether were from Dai Mo Chemical Co., Tianjin, China. Silica gel and silica gel GF254 plate were from Ocean Chemical Ltd. Qingdao, China. Automatic sample collector was from Shanghai Instrument Ltd, Shanghai, China.

4.3.2 METHODS

4.3.2.1 Isolation Method Contrast

4.3.2.1.1 Pressure Reducing Distillation (PRD)
100ml of ASVO was taken in a 250ml flask of the PRD instrument, and it was heated with the oil bath under low vacuum. When the oil bath temperature rose to 165°C, the distillation temperature reached 80°C and a large amount of yellow oil fraction appeared to be distilled, then the distillation temperature would drop to below 80°C. The oil bath temperature rose continuously to up to 170°C and the distillation temperature reached 116°C, a large amount of yellow oil fraction was distilled again and the distillation temperature would drop to below 116°C. The steps were repeated until the oil temperature rose to up to 190°C. Four different oil fractions at 80°C, 116°C, 118°C and 120°C of distillation temperature were obtained respectively.

4.3.2.1.2 Pressurized Column Chromatography (PCC)

The column was filled by silica gel as sorbent and the low toxicity solvent, petroleum ether and ethyl acetate were used as eluting solvent, elution isolation was performed under high pressure from the air pressure pump. The detailed procedures were as the follows;

**Column Making.** The column was made by the silica gel and petroleum ether, and pressed tightly by air pressure pump.

**Sample Loading.** ASVO was mixed up with silica gel adequately, and the mixture was taken to the top of the silica gel column.
**Eluting.** Eluted with the mixture solvent of specific volume ratio of petroleum ether and ethyl acetate and collected the eluate with an automatic sample collector.

**TLC Assay.** The TLC method of LIG could refer to **2.2.1.1**, and the LIG characteristic spot in the chromatogram showed visible blue fluorescence under 365 nm, and the $R_f$ of LIG was about 0.7. If there wasn’t any spot in the TLC chromatogram under the ultraviolet light at 254nm and 365nm, and also dyed with iodine vapor, it showed that the eluate contained LIG of high purity.

**Combination.** Combined the eluate with the high purity LIG, and the petroleum ether and ethyl acetate of the eluate were removed by rotary evaporator. Then the concentrated isolated LIG was obtained.

**4.3.2.1.3 Decompression Column Chromatography (DCC)**

Silica gel was used as sorbent, while petroleum ether and ethyl acetate were used as the eluting solvent. The DCC isolation was performed with buchner funnel as the isolation equipment. This method could realize rapid isolation conveniently under low pressure. The detailed procedures were as follows;

**Column Making.** The buchner funnel was filled with silica gel and the gel was condensed with a pressure reducing pump.

**Sample Loading.** ASVO was mixed up with silica gel adequately, and the mixture
was put on the top of the buchner funnel which had been filled with silica gel.

**Elution.** The mixture was eluted with a specific volume ratio of petroleum ether and ethyl acetate, and the eluate was collected sequentially.

**TLC Assay.** The TLC method of LIG could refer to 2.2.1.1, and the LIG characteristic spot in the chromatogram showed visible blue fluorescence under 365 nm, and the Rf of LIG was about 0.7. If there wasn’t any spot in the TLC chromatogram under the ultraviolet light at 254nm and 365nm, and also dyed with iodine vapor, it showed that the eluate contained LIG of high purity.

**Combination.** The eluate was combined with the high purity LIG, and the petroleum ether as well as ethyl acetate of the eluate was removed by a rotary evaporator. After that, the concentrated isolated LIG will be obtained.

The isolation condition could be adjusted appropriately each time base on the actual situation, such as changes the diameter of the buchner funnel, the polarity of the solvent or the amount of the samples collected until the purity of the isolated LIG met the requirement. This was a rapid isolation method. Moreover it could be stopped anytime without affecting the isolation effect.

### 4.3.2.1.4 Isolation Effect Contrast

The isolation effects of the three different methods were compared by using the
amount and purity of LIG as the index. The purity was examined by GC and HPLC. The result showed that DCC was the best isolation method for LIG. Further optimization was made on DCC, and a suitable process for industrial production was found.

4.3.2.2 Pilot Plant Test of DCC

Pilot plant test was performed by DCC. The mixture of 1000ml ASVO and 1kg silica gel (100-200 Mesh) was loaded in a preloaded column (diameter 30cm, height 45cm) with 8kg silica gel (300-400mesh). Then mixture was eluted and isolated according to the petroleum ether and ethyl acetate in the gradient proportions of 40:1, 30:1, 20:1, 10:1 and 5:1 in the decompression chromatography. The eluant was assayed with the TLC method, which used the silica gel GF$_{254}$ as the coating substance and a mixture of hexane-ethyl acetate (10:1) as the mobile phase. The TLC chromatogram was observed under 254nm and 365nm of ultraviolet light and dyed with iodine vapor. Only the eluants containing LIG spots were collected. They were further processed with condensation and purity assay.

4.3.2.3 Purity Assessment

4.3.2.3.1 TLC Assay
Please refer to 2.2.1.1.

4.3.2.3.2 HPLC Assay

Please refer to 2.2.1.2.

4.3.2.3.3 GC Assay

Please refer to 2.2.1.3.

4.3.2.4 Structure Assessment

4.3.2.4.1 GC-MS Assay

Please refer to 2.2.1.4.

4.3.2.4.2 NMR Assay

The instrument, Bruker 400MHz NMR spectrometer (Bruker Optics Co. Germany) was used for the NMR analysis. The isolated LIG sample was taken and dissolved into deuterium chloroform. After that, the $^1$H-NMR and $^{13}$C-NMR was examined by Bruker 400MHz NMR Spectrometer and the isolated LIG sample spectrum was
recorded.

4.3.2.4.3 UV Assay

Please refer to 2.2.1.5.

4.3.2.4.4 IR Assay

The instrument, Nicolet AVATAR 360 FT-IR spectrometer (Nicolet Vascular Inc. USA) was used for the IR analysis. The isolated LIG sample was taken and it was mixed with KBr. After that, the isolated LIG sample IR spectrum was examined and recorded by Nicolet AVATAR 360 FT-IR spectrometer.

4.4 RESULTS

4.4.1 Isolation Method Contrast (Table 4-1)

4.4.1.1 Pressure Reducing Distillation (PRD)

According to the PRD method, 100ml ASVO was taken in a 250ml flask and heated to isolate. Four fractions at different temperature were obtained: 1ml (80°C), 5ml (116 °C), 2ml (118°C) and 1ml (120°C). GC analysis revealed that the fractions at 116°C,
118°C and 120°C contained LIG, whereas, the purity were all below 90%, and the yield was low, only 1/3 when compared with silica gel column chromatography. The result showed that the isolation effect for LIG using this method was far from the expected result and not suitable for LIG isolation. This isolation time was about 4 hours. The result was shown in Table 4-1.

4.4.1.2 Pressurized Column Chromatography (PCC)

According to the PCC method, 40ml ASVO and 40g silica gel (100-200Mesh) mixture was load to the glass column (diameter 9cm, hight 80cm) which was filled with 1kg silica gel (300-400Mesh). Then the mixture was eluted with 18L petroleum ether and ethyl acetate (30:1) under the pressurization of membrane-type air pump and the products were collected sequentially. Subsequent thin layer chromatography detection using hexane - ethyl acetate (10:1) as the developer revealed that the products only contained a fraction of LIG. Combined the eluate with the high purity LIG, and the petroleum ether and ethyl acetate of the eluate were removed by a rotary evaporator. 6ml concentrated isolated LIG was obtained and GC analysis showed that the purity of LIG was 98%. The whole isolation time was about 40 hours. The result was shown in Table 4-1.

4.4.1.3 Decompression Column Chromatography (DCC)
According to the DCC method, 40ml ASVO and 40g silica gel (300-400Mesh) mixture was placed on the buchuer funnel (diameter 12.5cm) which was filled with 250g silica gel (300-400Mesh). After that, the mixture was eluted with 6L petroleum ether and ethyl acetate (30:1) under the pressure reducing and the products were collected sequentially. Subsequent thin layer chromatography detection using hexane - ethyl acetate (10: 1) as the developer revealed that the products only contained a fraction of LIG. Then the eluate was collected and GC analysis revealed that the purity of LIG was 98%. The whole isolation time was about 2 hours. The result was shown in Table 4-1.

**4.4.1.4 Isolation Effect Contrast**

In Table 4-1, the results showed that the purity of LIG which obtained by PRD was below 90% and the yield was very low. PRD was not an effective method to isolate LIG. However, the purity of the LIG obtained from PCC and DCC was above 98%. The amount and purity of the isolated LIG were equivalent in the two methods. However, the amount of eluate in DCC was much smaller than in PCC, and DCC took only 1/20 of the time of PCC. These results showed that the production efficiency of the LIG by DCC was much higher than the other two methods with a much lower cost. DCC was the optimal method to isolate LIG compared with other isolation methods.
The purity of the LIG obtained by the pilot plant test reached 98.1%, which showed that this method was available for large scale production of LIG. In addition, since its manipulation was more convenient, DCC was more suitable for industrial to amplify production. The method that LIG was isolated by using DCC had not been reported in the past, so we had applied the invention patent in China in 2005. Now the patent has been accredited.

4.4.2 Pilot Plant Test of DCC (Fig 4-1)

According to the pilot plant test method of DCC, 1000ml ASVO and 1kg silica gel (100-200Mesh) mixture was taken and it was placed in buchuer funnel (diameter 12.5cm) which was filled with 8g silica gel (300-400Mesh). Then, the mixture was eluted with petroleum ether and ethyl acetate in the gradient proportion of 40:1, 30:1, 20:1, 10:1 and 5:1 under pressure reducing. The eluant was assayed with the TLC method, and the chromatogram was observed under 254nm and 365nm of ultraviolet light and dyed with iodine vapor (Fig 4-1). The eluant containing only LIG spots was collected and condensed, for example the No.3 sample in Fig 4-1. For further assay, 153g slightly yellow liquid sample was finally obtained from isolated LIG of sample No.3 and the assay showed that the purity higher than 98%.

4.4.3 Purity Assessment
4.4.3.1 TLC Assay (Fig 4-2)

The TLC method was carried out, the methanol solution of isolated LIG sample was taken in the silica gel plate to develop, removed the plate, dried in air. After that, the plate was examined under ultraviolet light at 254nm and 365nm. The Plate was dyed with iodine vapour and the chromatogram was recorded (Fig 4-2). From the Fig 4-2, it showed that there was only one spot on different display conditions, also it was proved that the isolated LIG sample was a pure component.

4.4.3.2 HPLC Assay (Fig 4-3)

The HPLC method was carried out, the methanol solution of isolated LIG sample was injected, determined and the chromatogram was recorded (Fig 4-3). The content was calculated base on the percentage of the total peak area from the HPLC chromatogram analysis. From Fig 4-3, the retention time of the isolated LIG was 9.8 minute and the LIG content was 98.6%.

4.4.3.3 GC Assay (Fig 4-4)

The GC method was carried out, the isolated LIG sample was taken and injected. After the examined, the chromatogram was recorded (Fig 4-4). Also, the content was
calculated base on the percentage of total peak area from the GC chromatogram analysis. From Fig 4-4, the retention time of the isolated LIG was 12.4 minute and the LIG content was 98.3%.

4.4.4 Structure Assessment

4.4.4.1 GC-MS Assay (Fig 4-5, 4-6)

The GC-MS method was carried out, the isolated LIG sample was taken and was injected. After examined, the total ion chromatogram and the mass spectrum of main peak were recorded (Fig 4-5, 4-6). From Figs 4-5 and 4-6, the retention time of the isolated LIG was 8.4 minute and the LIG content was 98.4%. The mass spectrum of the isolated LIG sample showed that the molecule ion peak was m/z 190.

4.4.4.2 NMR Assay (Fig 4-7, 4-8)

The isolated LIG sample was taken and dissolved into deuterium chloroform. After that, the sample was examined by 1H-NMR and 13C-NMR with the Bruker 400MHz NMR Spectrometer and the spectrum was recorded (Fig 4-7, 4-8).

4.4.4.3 UV Assay (Fig 4-9)
The isolated LIG sample was taken and dissolved into methanol. The sample was examined by 190-600nm UV spectrum with Lambda35 UV/VIS spectrometer and the spectrum was recorded (Fig 4-9).

4.4.4.4 IR Assay (Fig 4-10)

The isolated LIG sample was taken and mixed with KBr. The mixture was examined by Nicolet AVATAR 360 FT-IR spectrometer and the IR spectrum was recorded (Fig 4-10).

From Figs 4-6, 4-7,4-8,4-9 and 4-10, carried on the analysis of the chromatograms, and the all results were as follows:

EI-MS m/z:  190(M⁺), 161(M⁺-C₂H₅), 148, 133(M⁺-C₃H₇-CO), 106, 77, 55, 27

¹H-NMR(CDCl₃)δ:  0.96(t, J=8,3H), 1.52(m, J=8,2H),  2.38-2.63(m,6H), 5.24(t, J=8, 1H), 6.01(m, J=8, 1H) 6.29(d, J=8, 1H)

¹³C-NMR(CDCl₃)δ :  13.67(-CH₃), 18.45(-CH₂-), 22.32(-CH₂-), 22.36(-CH₂-), 28.05(-CH₂-), 112.73(C=), 117.01(C=), 123.92(C=), 129.83(C=), 146.99(C=), 148.54(0-C=), 167.46(0-C=0)

UVλmax(MeOH)nm:  330, 280, 210

IR(KBr)υcm⁻¹:  3050(C=C-H), 2960, 2933, 2872(CH₂, CH₃), 1766, 1688(C=C), 1437, 1271, 1050(0-C=0), 706
From the above data, we know that the main functional group of the sample structure included -CH3, -CH2-, C=, O-C=, O-C=O and so on, and the data was consistent with the reports of Z-ligustilide(Z-LIG) (Hu et al., 2003; Liu et al., 2004; Liu et al., 2003). So it was proved that the prepared compound was LIG. The chemical and 3-D structure of Z-LIG was shown in Fig 4-11 and Fig 4-12.

4.5 DISCUSSION

Gravity silica gel column chromatography is a commonly used method for LIG isolation. However, it is time-consuming and reagents-consuming with a rather low efficiency. A comparative study was carried out in present study by using PRD, PCC, and DCC respectively. Based on the characteristics of LIG, they had different efficacy of separation. PRD separated the substances at different boiling points and sharply reduced the separation time, but it had a low purity of LIG, because some other components had similar boiling points. PCC was less time-consuming than gravity silica gel column chromatography and had a good efficacy of separation, but it only served for experimental level preparation due to its complicated operation and reagents-consuming procedures. DCC was the most effective and convenient method for LIG separation, with an approximate 98% purity. This study enlarged LIG separation to pilot plant scale. Results showed a purity of 98.1%, indicating that this kind of separation method was suitable for large-scale production. DCC for LIG separation had several advantages. Firstly, there were simple operation, simple
equipments, rapid separation speed and high recovery rate. Then, it had the lowest requirement for materials as well as solvents, and could save costs and contribute to environmental protection. Third, there is no time restriction, e.g., it could be stopped anytime without affecting the isolation effect. Lastly, its separation scale could be enlarged to make it completely appropriate for industrialized large-scale production. Thus, this study has broken the technical bottleneck of the industrialized separation of LIG. It is the first report on the separation of LIG by using this kind of method, also the first report to realize the large-scale production of LIG. We had applied the invention patent in China in 2005. Now the patent has been accredited.

The selection of eluant is very important in column chromatography separation. While choosing an eluant, the characteristics of both the separated component and the chosen sorbent should be considered together. When a polar sorbent is used in chromatography and the polarity of the separated component is slight, a slight polar solvent should be chosen as the eluant. Meanwhile, a polar solvent should be chosen if the separated component is a strong polar compound. Due to the low polarity of LIG in this study, the mixture of petroleum ether and ethyl acetate in fixed proportion was chosen. In the experiment of LIG separation by column chromatography, a gradient method was introduced in the elution procedure in order to get a high LIG purity. The polarity of the eluant was increased progressively at a certain speed, making each component which was adsorbed on chromatographic column eluted one by one. But the increase of polarity should not be too sharp, or it would lead to
unsatisfactory separation results. Therefore, it was not proper to randomly combine two solvents with significant different polarity, or to randomly increase the amount of the strong polar solvent. In this study, the eluant was prepared with petroleum ether and ethyl acetate in different proportions of 40:1, 30:1, 20:1, 10:1 and 5:1, all of which showed good efficacy of separation.

A suitable collection and combination of the eluant was the key procedure in the process of separating LIG. Accordingly, a simple and quick qualitative analysis was always applied before the combination of the eluant, which was very important and had obvious effect on the LIG purity. The experiment indicated TLC was the best qualitative identification method for fast collection and combination of the eluant.

In the LIG separation, if the eluant of LIG purity turned out to be unqualified, it must be incorporated into other solutions containing LIG and impurities. And then the LIG was completely re-separated by repeating the separation steps, methods and conditions, just as what had been carried out at the first DCC separation. Generally, the samples after each re-separation contain fewer impurities, also showing a high speed and good efficiency during the re-separation. Separation could be recycled for several times in both experiment and manufacturing to get the required high purity of LIG.

In this experiment, the peak area normalization method of chromatogram was used for
the purity assay of LIG. Under certain chromatographic conditions, the concentration of the components in mobile phase showed a positive correlation with the peak area or peak height of the response signals of the detectors. Since the size of the peak area was not subject to influences from the operating conditions, such as column temperature, flow velocity of mobile phase, and sample introduction rate, the peak area was more appropriate for a parameter of quantitative analysis. Taking the sum of all the components as 100%, and their related response signals as quantitative parameters, the normalization method could calculate results easily and accurately, especially for those liquid samples whose volume were difficult to measure accurately because of their small amount of sample introduction. HPLC, GC and GC-MS used in present experiment were equipped with an electric integrating instrument to accurately measure the peak area, so the report from detecting system was directly based on the percentage of the total peak area by every analysis method.

The above result of the percentage of total peak area showed the content of LIG was 98.4% by GC-MS assay, while 98.3% by GC and 98.6% by HPLC, respectively. There was a slightly difference among the results of the three assaying methods because of systematic errors caused by the different apparatuses, this wouldn’t affect the final conclusion. The above results of LIG content indicated that the purity of the prepared LIG was over 98% and could be used as the reference of LIG as well as raw materials for the new drug development of LIG.
Table 4-1. Isolation method contrast of LIG.

<table>
<thead>
<tr>
<th>Isolation method</th>
<th>ASVO (ml)</th>
<th>Silica gel (kg)</th>
<th>Eluate (L)</th>
<th>Time (h)</th>
<th>LIG volume (ml)</th>
<th>LIG purity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRD</td>
<td>100</td>
<td></td>
<td></td>
<td>4</td>
<td>9</td>
<td>90</td>
</tr>
<tr>
<td>PCC</td>
<td>40</td>
<td>1.0</td>
<td>18</td>
<td>40</td>
<td>6</td>
<td>98.5</td>
</tr>
<tr>
<td>DCC</td>
<td>40</td>
<td>0.25</td>
<td>6</td>
<td>2</td>
<td>6</td>
<td>98.2</td>
</tr>
</tbody>
</table>

Figure 4-1. The TLC chromatogram of the eluant (left: ultraviolet light 254nm, right: ultraviolet light 365nm, underside: Dyed with iodin vapor). In this TLC chromatogram, No.3 sample contained high purity LIG. It was then collected separately, condensed, and assayed, showing a purity of more than 98%. No. 2 and No. 4 also contained a certain amount of LIG with a rather low purity, and they could be separated again after incorporation and collection. Samples of other numbers needed no further treatment because the concentration of LIG was very low.
Figure 4-2. The TLC chromatogram of the isolated LIG sample (left: ultraviolet light 254nm, middle: ultraviolet light 365nm, right: Dyed with iodine vapor). The spots of each picture were the high and low concentration of the isolated LIG form left to right respectively. It showed that there was only one point on different wavelength conditions, and it was proved that the isolated LIG was purity component.

Figure 4-3. The HPLC chromatogram of the isolated LIG. The retention time of the isolated LIG was 9.8 minute and the LIG content was 98.6%.
Figure 4-4. The GC chromatogram of the isolated LIG. The retention time of the isolated LIG was 12.4 minute and the LIG content was 98.3%.

Figure 4-5. The total ion chromatogram of the isolated LIG in GC-MS. The retention time of the isolated LIG was 8.4 minute and the LIG content was 98.4%.
Figure 4-6. The mass spectrum of the isolated LIG. The molecule ion peak was m/z 190, EI-MS m/z: 190 (M⁺), 161 (M⁺-C₂H₅), 148, 133 (M⁺-C₂H₅-CO), 106, 77, 55, 27
Figure 4-7. The $^1$H–NMR spectrum of the isolated LIG. $^1$H-NMR(CDCl$_3$)$\delta$: 0.96(t, $J$=8, 3H), 1.52(m, $J$=8, 2H), 2.38-2.63(m, 6H), 5.24(t, $J$=8, 1H), 6.01(m, $J$=8, 1H), 6.29(d, $J$=8, 1H)
Figure 4-8. The $^{13}$C-NMR spectrum of the isolated LIG. $^3$C-NMR(CDCl$_3$)$\delta$: 13.67(-CH$_3$), 18.45(-CH$_2$), 22.32(-CH$_2$), 22.36(-CH$_2$), 28.05(-CH$_2$), 112.73(C=), 117.01(C=), 123.92(C=), 129.83(C=), 146.99(C=), 148.54(0-C=), 167.46(0-C=0)
Figure 4-9. The UV spectrum of the isolated LIG. UV$\lambda_{\text{max}}$(MeOH) nm: 330, 280, 210

Figure 4-10. The IR spectrum of the isolated LIG. IR(KBr) $\nu_{\text{cm}^{-1}}$: 3050 (C=C-H), 2960, 2933, 2872 (CH$_2$, CH$_3$); 1766; 1688 (C=C); 1437, 1271, 1050 (C-O-C), 706
Figure 4-11. The chemical structure of Z-ligustilide

Figure 4-12. The 3-D structure of Z-ligustilide
CHAPTER 5

PROPERTY AND STABILITY STUDY OF LIGUSTILIDE

5.1 ABSTRACT

In this experiment, the physical and chemical properties of LIG, including appearance, boiling point, density, solubility and lipid-water distribution coefficient were investigated by using high purity LIG (>98%). The affecting factors of LIG stability were tested by accelerating test under extraordinary condition. These factors which included temperature, light, oxygen, oxidant, reductant, acidity, alkalinity, metal ion and antioxidant were studied systematically. It showed that LIG was a slightly yellow oily liquid with a fresh sweet scent. It had a little astringent taste and was tongue-numbing. The normal boiling point, density, water solubility and lipid-water distribution coefficient of LIG were 296-299°C, 0.979 ±0.005 g/ml, 0.004±0.0002% (w/w) and 3790±185 respectively. The results indicated that temperature, light, and oxygen had great influence on the stability of LIG. LIG was extremely unstable in acidic condition or with the influence of reductant. Antioxidants could markedly improve the stability of LIG. These conclusions could provide a good foundation for the new drug development of LIG, especially for its pharmaceutical preparation.
5.2 INTRODUCTION

Up to now, there have been few systematic studies on the physical and chemical properties of LIG. The density, solubility and lipid-water distribution coefficient of LIG have not been exactly reported. These physical properties will greatly help the study on the stability, manufacturing, package, storage and clinical application of LIG. In this experiment, with high purity LIG as the raw substance, systematic and comprehensive studies on the physical and chemical properties of LIG were carried out.

It was reported that the stability of LIG was greatly improved in solvent. The LIG purity was decreased from 97.98% to 96.36% in chloroform, and to 91.24% in hexane at room temperature within 25 days, which indicated LIG in organic solvent was stable (Zhou et al., 2001). This was attributed to the salvation effect of the solvent which had similar polarity with LIG, and also increased the solvate energy in solute and solvent reacting system. Raising activation energy and lowering total energy could provide higher stability of LIG. So LIG could remain stable in solvent (Li et al., 2005). The LIG stability test in methanol in Chapter 3 showed that methanol was a good solvent for LIG, as LIG possessed good stability in methanol without any obvious content variations after being placed at room temperature for 48 h, and methanol was also a common solvent with temperate polarity. Therefore we adopted
methanol as the solvent of LIG in the examinations of the stability and the quantitative determination of LIG with HPLC.

The chemical structure of LIG is a typical structure of phthalide, and there is an active butenyl group on the third position of LIG. LIG is extremely unstable at room temperature, and is easily isomerized to other kinds of 3-alkylphthalide through oxidation, isomerization, dimerization, etc (Zhou et al., 2001). For long-term storage, it might be oxidized into various complicated productions, which caused inconvenience to the preparation and storage of LIG. It was reported that about 58% of LIG was isomerized at room temperature with protection from light after a 15-day storage, while a longer shelf life could be obtained at -20°C (Zhou et al., 2001). In our separation experiment we also found that LIG was very likely to isomerize due to its extremely poor stability. Because of its instability, LIG can change into other phthalide compounds, making its preparation and storage very difficult, also its research and development highly restricted. Therefore, it is of great importance to investigate the factors influencing the stability of LIG. In this experiment, LIG of high purity was applied as a raw substance, and then systematic investigations were carried out on various factors affecting the LIG stability. The results would provide a good foundation for the new drug development of LIG.

5.3 MATERIALS AND METHODS
5.3.1 Materials

5.3.1.1 Materials

Ethyl acetate, hydrochloric acid and sodium hydroxide were from Beijing Chemical Co., Beijing, China. Methanol, hexane and petroleum ether were from Dai Mo Chemical Co., Tianjin, China. Silica gel GF$_{254}$ plate was from Ocean Chemical Ltd. Qingdao, China. Ferrous chloride, ascorbic acid, potassium chloride, aluminium oxide, magnesium sulfate, sodium chloride, Cupric Sulfate, Ferrous Sulfate and Zinc Chloride were from Sigma Chemical Co. USA. Vitamin E was from BASF Chemical Co. Germany. HPLC grade methanol was purchased from Honeywell International Inc., Muskegon, USA. HPLC grade isopropyl alcohol was from Tedia Company, Fairfield, USA. H$_2$O$_2$(30%, w/v) was from the Riedel-de Haen, Germany. Deionized water was generated from a Milli-Q water system (Millipore, Bedford, MA, USA).

5.3.1.2 LIG Preparation

LIG was extracted, separated and purified from Radix *Angelica sinensis* in our laboratory, with batch number: 050118. Purified LIG was identified by electron impact ionization (EI) MS, H$^1$ NMR and C$^{13}$ NMR spectrometric techniques. The purity of LIG was found to be > 98% based on the percentage of total peak area by GC assay.
5.3.2 METHODS

5.3.2.1 HPLC Assay of LIG

Please refer to 2.2.3.1.

5.3.2.2 Property Testing of LIG

5.3.2.2.1 Appearance Assessment

The color, smell and taste of LIG were investigated with high purity LIG.

5.3.2.2.2 Determination of Boiling Point

Please refer to 2.2.5.1.

5.3.2.2.3 Determination of Density

Please refer to 2.2.5.2.
5.3.2.2.4 Determination of Solubility

Please refer to 2.2.5.3.

5.3.2.2.5 Determination of Lipid-water Distribution Coefficient

Please refer to 2.2.5.4.

5.3.2.3 Affecting Factors Testing for LIG stability

5.3.2.3.1 Effect of Temperature

The LIG was taken into the small centrifuge tubes, and the nitrogen was filled into the tubes to expel oxygen. After that, the tubes were sealed and wrapped with tinfoil paper. The processed centrifuge tubes were placed in an oven at 40, 60 and 80°C for 48h respectively, and taken out at 6, 12, 24 and 48 h. The residual content of LIG at each time point was determined by the above HPLC method and compared with the content at 0 h to evaluate its stability under different temperature without oxygen and light influence.

5.3.2.3.2 Effect of Oxygen
The LIG was taken into the small centrifuge tubes, and each tube was filled with nitrogen, oxygen and air respectively. After that, the tubes were sealed and were wrapped with tinfoil paper. The processed centrifuge tubes were placed in an oven at 40°C for 48h, and taken out at 6, 12, 24, and 48 h respectively. The residual content of LIG at each time point was determined by the above HPLC method and compared with the content at 0 h to evaluate its stability in different air at the same temperature without any influence of light.

5.3.2.3.3 Effect of Light

The LIG was taken into the small centrifuge tubes and the tube was filled with nitrogen to expel the oxygen. After that, the tubes were sealed. The processed centrifuge tubes were placed in darkness, natural light and a 60W daylight lamp under room temperature for 8 day, and taken out at 1, 2, 4 and 8 day respectively. The residual content of LIG at each time point was determined by the above HPLC method and compared with the content at 0 day to evaluate its stability in different light at the same temperature without any influence of oxygen.

5.3.2.3.4 Effect of Oxidant and Reductant

20mg of LIG was taken and placed in 100ml volumetric flask, which was dissolved and diluted with methanol to volume. After that, the solution was mixed well and
used as the standard solution. H₂O₂ and Na₂SO₃ were used as the oxidant and the reductant respectively. 5%, 10% and 20% solution of H₂O₂ and Na₂SO₃ were prepared respectively, and then 2% of every solution of H₂O₂ and Na₂SO₃ was added into a LIG standard solution tube, respectively. The tubes were placed in darkness under room temperature. One set of solution sample tubes was added Na₂SO₃ at 1, 2, 4 and 8 h respectively, as the other set of solution sample tubes was added H₂O₂ at 6, 12, 24 and 48h respectively. The residual content of LIG at each time point was determined by the above HPLC method and compared with the content at 0 h to evaluate its stability in each concentration of oxidant and reductant.

5.3.2.3.5 Effect of Acidity and Alkalinity

20mg of LIG was taken and placed in 100ml volumetric flask, which was dissolved and diluted with methanol to volume. After that, the solution was mixed well and used as the standard solution. 1%, 2% and 4% of HCl and NaOH were prepared respectively, and then 2% of every solution of HCl and NaOH was added into a LIG standard solution tube respectively, and placed the tubes in darkness under room temperature. One set of solution sample tubes was added HCl at 1, 2, 4 and 8 h respectively, as the other set of solution sample tubes was added NaOH at 6, 12, 24 and 48h respectively. The residual content of LIG at each time point was determined by the above HPLC method and compared with the content at 0 h to evaluate its stability in each different concentration acidity and alkalinity.
5.3.2.3.6 Effect of Metal ion

20mg of LIG was taken and placed in 100ml volumetric flask, which was dissolved and diluted with methanol to volume. After that, the solution was mixed well and used as the standard solution. 2% of the common metal ion solutions including ferrous chloride, potassium chloride, aluminium oxide, magnesium sulfate, sodium chloride, Cupric Sulfate, Ferrous Sulfate and Zinc Chloride, were prepared respectively. After that, 2% of every solution was added into a LIG standard solution tube respectively. Then the tubes were placed in darkness at room temperature for maximum 48h, and the tubes were taken out at 6, 12, 24 and 48h respectively. The residual content of LIG at each time point was determined by the above HPLC method and compared with the content at 0 h to evaluate its stability in each different metal ion.

5.3.2.3.7 Effect of Antioxidant

20mg of LIG was taken and placed in 100ml volumetric flask, which was dissolved and diluted with methanol to volume. After that, the solution was mixed well and used as the standard solution.5% and 10% of Vitamin E and Vitamin C were prepared respectively, and then 2% of every solution of Vitamin E and Vitamin C was added into a LIG standard solution tube, respectively, which was then sealed and placed in an oven at 50°C for 48h, and taken out at 6, 12, 24 and 48 h respectively. The content
of LIG at each time point was determined by the above HPLC method and compared with the content at 0 h to evaluate its stability in each different concentration of antioxidant.

5.4 RESULTS

5.4.1 Property Testing of LIG

5.4.1.1 Appearance Assessment (Fig5-1)

The appearance of LIG was investigated with high purity (Fig5-1). The result indicated that LIG was a slightly yellowish oily liquid with a fresh sweet scent and a little astringent taste and gave a tongue-numbing feeling.

5.4.1.2 Determination of Boiling Point

Microdose Ebulliometry

According to the method of microdose ebulliometry, taken 1 drop of LIG to the outer tunnel of the microdose tube, nitrogen was filled into the tube. The boiling point was measured for 3 times in the oil bath at high temperature. The result indicated the mean boiling point of LIG was 296-299°C.
Vacuum Distillation

According to the method of vacuum distillation, 20ml of LIG was taken and placed in a 50ml flask which filled with nitrogen. After that, the LIG was heated and determined the boiling point for 3 times when the vacuum achieved 6 mmHg. The result indicated that the mean boiling point under 6 mmHg vacuum of LIG was 166-169℃/6mmHg.

5.4.1.3 Determination of Density

According to the method of density testing, LIG and water was taken into 20ml clean and dry picnometer respectively. After that, the picnometer was placed in water bath of constant temperature at 30℃ for 30 min. Then, the picnometer was taken out, determined and calculated. Testing was repeated for 3 times. The result indicated that the measured density of LIG was 0.979 ± 0.005 g/ml, which was a little lighter than water.

5.4.1.4 Determination of Solubility

According to the method of density testing, 50mg, 100mg and 200mg was taken and placed into the stopple-matched standard test tube respectively. After that, 5ml water was added to sonication for 5 min, and it was placed in a swing bed for 100 r/min pendulation at 25℃ for 24 h. The tubes were taken out and underwent centrifugation.
The water in the underlayer was sucked and then filtered by a micropore filtration film. The filtrate was diluted and the content of LIG was determined using HPLC. Testing was repeated for 3 times. The result indicated that the LIG solubility in water was 0.004±0.0002% (w/w).

5.4.1.5 Determination of Lipid-water distribution coefficient

According to the method of lipid-water distribution coefficient testing, 1ml of phosphate buffer and 1ml n-octanol were mixed. After that, 0.1g LIG was added to the mixture and then it was placed in a swing bed at constant temperature (37°C) for pendulation (100 r/min). Then, the mixture was taken out and let it stand demixing after 8 h later. After separation, the LIG concentration in two phases was determined by the above HPLC method. Testing was repeated for 3 times. The result indicated that the lipid-water distribution coefficient of LIG was 3790±185.

5.4.2 Affecting Factors Testing of LIG stability

5.4.2.1 Effect of Temperature (Table 5-1 and Fig 5-2)

According to the method of temperature accelerated testing, the LIG samples was taken out at each time point and the residual content was determined by HPLC which compared with the content at 0 h to evaluate its stability under different temperature
without oxygen and light influence. The effects of temperature were shown in Table 5-1 and Fig 5-2. The content of LIG was remarkably decreased as the temperature increased, about 13% and 79% of LIG remained when heated at 80°C and 40°C, respectively for 48hrs. It proved that the stability of LIG was influenced fatally by high temperature. Thus LIG should be conserved at as low temperature as possible.

5.4.2.2 Effect of Oxygen (Table 5-2 and Fig 5-3)

According to the method of oxygen accelerated testing, the LIG samples was taken out at each time point and the residual content was determined by HPLC, and compared with the content at 0 h to evaluate its stability in different air at the same temperature without any influence of light. The effects of oxygen were shown in Table 5-2 and Fig 5-3. With an increased concentration of oxygen at 40°C, the content of LIG was decreased when the influence of light was absent. The content of LIG was 26% and 35% when it was in oxygen and in air respectively for 48hrs. This indicated that the stability of LIG was obviously affected by oxygen. Thus, it could be concluded that oxygen played an important role in the oxidation reactions of LIG.

5.4.2.3 Effect of Light (Table 5-3 and Fig 5-4)

According to the method of light accelerated testing, the LIG samples was taken out at each time point and the residual content was determined by HPLC which compared
with the content at 0 day to evaluate its stability in different light at the same room temperature without any influence of oxygen. The effects of light were shown in Table 5-3 and Fig 5-4. Only 18% of LIG remained when LIG was exposed to a 60W daylight lamp without any influence of oxygen for 8 days. However, over 98% of LIG remained when it was placed in the dark for 8 days. Thus, it proved that LIG was very sensitive to light and it could cause the degradation of LIG. This conclusion proved that photo degradation was a major degradation pathway. This indicated that LIG needed to be strictly protected from light.

5.4.2.4 Effect of Oxidant and Reductant (Table 5-4, 5-5 and Fig 5-5, 5-6)

According to the method of oxidant and reductant accelerated testing, 2% of each solution H₂O₂ and Na₂SO₃ were added into the LIG standard solution tube respectively. After that, the tubes were placed without light and the LIG samples were taken at each time point. The residual content of LIG was determined by HPLC which compared with the content at 0 h to evaluate its stability in each different concentration of oxidant and reductant. The results were in Tables 5-4, 5-5 and Figs 5-5, 5-6. The content of LIG was not obviously decreased when H₂O₂ was added into the LIG standard solution, and the content was 85.7% in 20% of H₂O₂ for 48hrs. This indicated that the oxidant had a little influence on the LIG stability. However, soon after different concentrations of Na₂SO₃ were added, the measured LIG content fell quickly to above 50% in one hour, and a new peak was appeared on the HPLC
chromatogram, this showing that the production degraded of LIG. This indicated that Na$_2$SO$_3$ maybe included other reductants could react with LIG to produce new compounds. Almost all LIG was degraded in 5%, 10% and 20% of Na$_2$SO$_3$ after 8h. The content of LIG was only 3.7% in 20% of Na$_2$SO$_3$ after 8h. The results indicated that LIG was extremely unstable in the reductant condition and could react with reductant to produce new compounds. The characteristic of LIG was specially influenced the application and development of it.

5.4.2.5 Effect of Acidity and Alkalinity (Tables 5-6, 5-7 and Figs 5-7, 5-8)

According to the method of acid and alkali accelerated testing, 2% of each solution HCl and NaOH were added into the LIG standard solution tube respectively. After that, the tubes were placed without light and the LIG samples were taken at each time point. The residual content of LIG was determined by HPLC which compared with the content at 0 h to evaluate its stability in each different concentration acid and alkali. The results were in Tables 5-6, 5-7 and Figs 5-7, 5-8. The content of LIG was not obviously decreased when NaOH was added into the LIG standard solution, and the content was 87.3% in 4% of NaOH for 48hrs. The results indicated that alkalinity had a little influence on the LIG stability. However, the measured content of LIG was only 4.1% and 1.3 % in 1%, 2% of HCl for 8h respectively, and all LIG disappeared in 4% of HCl for 8hrs. This indicated that LIG was hydrolyzed rapidly by a high concentration of acid. When the time of LIG in acid was increasing, some new peaks
appeared and then disappeared on the HPLC chromatogram. This proved that acidity had influenced fatally to LIG. Thus LIG must be kept away from acid in the pharmaceutical preparation and application of LIG.

5.4.2.6 Effect of Metal Ion (Table 5-8 and Fig 5-9)

According to the method of acid and alkali accelerated testing, 2% of 8 metal ion solutions were added into the LIG standard solution tube respectively. After that, the tubes were placed without light and the LIG samples were taken at each time point. The residual content of LIG was determined by HPLC which compared with the content at 0 h to evaluate its stability in each different metal ion. The results were in Table 5-8 and Fig 5-9. The content of LIG decreased a little when metal ions solution was added into the LIG standard solution, and the lowest content of LIG was 82.7% in ferrous chloride after 48h. The results indicated that the common metal ions had no obvious effect on the LIG stability.

5.4.2.7 Effect of Antioxidant (Table 5-9 and Fig 5-10)

According to the method of antioxidant accelerated testing, 2% of each solution Vitamin E and Vitamin C were added into the LIG standard solution tube respectively. After that, the tubes were placed in oven at 50°C without light and the LIG samples were taken at each time point. The residual content of LIG was determined by HPLC
which compared with the content at 0 h to evaluate its stability in each different concentration antioxidant. The results were in Table 5-9 and Fig 5-10. After the LIG standard solution with Vitamin E was heated at 50°C for 48hrs, the content of LIG was over 95%. This indicated that Vitamin E could protect LIG and improve its stability. On the other hand, the LIG standard solution with Vitamin C was heated at 50°C for 48hrs, the content of LIG only had 65%. This indicated that Vitamin C, being the acid component, could accelerate LIG degradation because LIG was very unstable in the acidic condition.

5.5 DISCUSSION

A comprehensive investigation on the physical and chemical properties of LIG was carried out in this experiment. LIG was a slightly yellow oily liquid with a fresh sweet scent and gave a little astringent taste and tongue-numbing feeling. The measured density of LIG was 0.979 ±0.005 g/ml, which was slightly lower than water. The water solubility of LIG was 0.004±0.0002% (w/w), and it was very soluble in organic solvents such as ethanol, methanol, acetic ether, ethyl acetate, petroleum ether, etc. The measured normal boiling point of LIG was 296-299°C, and the vacuum boiling point was 166-169°C/6mmHg. It was revealed that LIG was a highly liposoluble substance, with a lipid-water distribution coefficient of 3790±185. These results of LIG properties could provide a good foundation for the development of LIG.
Comparison studies were conducted to explore the factors affecting the stability of LIG. Temperature, light and oxygen all turned out to have an obvious influence on the stability of LIG, thus the protection from high temperature, light and oxygen was needed as much as possible for the conservation of LIG. LIG was extremely unstable in acidic and reductant conditions and could react with acid and reductant to produce new compounds. Thus LIG must be kept away from acid and reductant in the pharmaceutical preparation and application. Oxidation, hydrolysis, and photodegradation were shown to be the major degradation routes of LIG. The characteristic of LIG had specially influenced the application and development of it. The 8 kinds of common metal ions had no obvious effect on LIG’s stability, so there was no need to pay special attention to this aspect. Antioxidant could improve the stability of LIG, and the use of Vitamin E was a great help to minimize the decomposition of LIG. This provided a good foundation for improving LIG’s stability and designing new preparations, and the results could be further applied to other cases.
Figure 5-1. Samples of high purified LIG. LIG is a slightly yellow oily liquid with a fresh sweet scent and gives a little astringent taste and tongue-numbing feeling.
**Table 5-1.** The results of temperature effect.

(Mean ± S.D. of three measurements)

<table>
<thead>
<tr>
<th>Temperature</th>
<th>6h</th>
<th>12h</th>
<th>24h</th>
<th>48h</th>
</tr>
</thead>
<tbody>
<tr>
<td>40°C</td>
<td>94.5±0.4</td>
<td>88.6±0.3</td>
<td>82.3±1.1</td>
<td>79.1±0.4</td>
</tr>
<tr>
<td>60°C</td>
<td>88.9±0.6</td>
<td>76.8±1.2</td>
<td>69.0±1.2</td>
<td>58.9±0.7</td>
</tr>
<tr>
<td>80°C</td>
<td>67.7±0.7</td>
<td>55.6±0.5</td>
<td>33.8±0.4</td>
<td>13.0±0.2</td>
</tr>
</tbody>
</table>

**Figure 5-2.** The results of temperature effect. The content of LIG was decreased remarkably as heating temperature increased, about 13% and 79% of LIG remained when heated at 80°C and 40°C for 48hrs, respectively. It proved that the stability of LIG was influenced fatally by high temperature. Thus LIG should be conserved at as lower temperature as possible.
Table 5-2. The results of oxygen effect

(Mean ± S.D. of three measurements)

<table>
<thead>
<tr>
<th></th>
<th>6h</th>
<th>12h</th>
<th>24h</th>
<th>48h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrogen</td>
<td>94.5±0.8</td>
<td>88.6±1.2</td>
<td>82.3±1.4</td>
<td>79.1±0.3</td>
</tr>
<tr>
<td>Air</td>
<td>82.1±1.1</td>
<td>62.3±0.9</td>
<td>54.3±0.8</td>
<td>43.9±0.9</td>
</tr>
<tr>
<td>Oxygen</td>
<td>51.4±1.9</td>
<td>33.1±0.7</td>
<td>28.5±0.6</td>
<td>25.8±0.8</td>
</tr>
</tbody>
</table>

Figure 5-3. The results of oxygen effect. With an increasing concentration of oxygen at 40°C, the content of LIG was decreased when the influence of light was avoided. The content of LIG was 26% and 35% when the LIG was in oxygen and in air for 48hrs respectively. It indicated that the stability of LIG was obviously affected by oxygen. Thus, it could be concluded that oxygen played an important role in the oxidation reactions of LIG.
Table 5-3. The results of light effect

(Mean ± S.D. of three measurements)

<table>
<thead>
<tr>
<th></th>
<th>1d</th>
<th>2d</th>
<th>4d</th>
<th>8d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Darkness</td>
<td>99.8±0.5</td>
<td>99.3±0.4</td>
<td>98.6±0.6</td>
<td>98.0±0.3</td>
</tr>
<tr>
<td>Natural light</td>
<td>92.1±0.6</td>
<td>82.4±1.4</td>
<td>69.3±1.3</td>
<td>51.5±1.4</td>
</tr>
<tr>
<td>Daylight lamp</td>
<td>68.9±1.9</td>
<td>51.3±1.5</td>
<td>30.1±0.9</td>
<td>18.5±1.0</td>
</tr>
</tbody>
</table>

Figure 5-4. The results of the effect of light. When LIG was exposed to a 60W lamp for 8 days, only 18% of LIG remained. However, when placed in darkness for 8 days, over 98% of LIG remained. Thus it proved that LIG was very sensitive to illumination.
Table 5-4. The results of oxidant effect

(Mean ± S.D. of three measurements)

<table>
<thead>
<tr>
<th></th>
<th>6h</th>
<th>12h</th>
<th>24h</th>
<th>48h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pure LIG</td>
<td>99.8±0.4</td>
<td>99.6±0.8</td>
<td>99.2±0.7</td>
<td>98.8±1.1</td>
</tr>
<tr>
<td>5%H₂O₂</td>
<td>93.1±0.8</td>
<td>92.1±1.4</td>
<td>90.8±1.1</td>
<td>90.1±1.3</td>
</tr>
<tr>
<td>10%H₂O₂</td>
<td>92.4±1.6</td>
<td>91.4±0.7</td>
<td>90.2±1.5</td>
<td>88.9±1.0</td>
</tr>
<tr>
<td>20%H₂O₂</td>
<td>88.9±1.3</td>
<td>88.1±0.4</td>
<td>87.3±0.7</td>
<td>85.7±0.9</td>
</tr>
</tbody>
</table>

Figure 5-5. The results of the effect of oxidant. The content of LIG was not obviously decreased when H₂O₂ was added into the LIG standard solution, and the content was 85.7% in 20% of H₂O₂ for 48hrs. It indicated that the oxidant had a little influence on the LIG stability.
Table 5-5. The results of reductant effect

(Mean ± S.D. of three measurements)

<table>
<thead>
<tr>
<th></th>
<th>1h</th>
<th>2h</th>
<th>4h</th>
<th>8h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pure LIG</td>
<td>100±0.6</td>
<td>99.9±0.3</td>
<td>99.8±0.7</td>
<td>99.8±0.4</td>
</tr>
<tr>
<td>5%Na₂SO₃</td>
<td>57.6±1.3</td>
<td>25.1±0.4</td>
<td>15.9±0.7</td>
<td>6.8±0.2</td>
</tr>
<tr>
<td>10%Na₂SO₃</td>
<td>45.8±1.4</td>
<td>19.3±0.8</td>
<td>12.6±0.5</td>
<td>5.2±0.3</td>
</tr>
<tr>
<td>20%Na₂SO₃</td>
<td>35.8±0.6</td>
<td>15.3±0.2</td>
<td>8.9±0.3</td>
<td>3.7±0.2</td>
</tr>
</tbody>
</table>

Figure 5-6. The results of the effect of reductant. LIG was almost all degraded in 5%, 10%, 20% of Na₂SO₃ after 8h respectively. The content of LIG was 3.7% in 20% of Na₂SO₃ after 8h, and a new peak was appeared on the HPLC chromatogram, this showing that the production degraded of LIG. The results indicated that LIG was extremely unstable in the reductant condition and could react with reductant to produce new compounds. The characteristic of LIG was specially influenced to the application and development of LIG.
Table 5-6. The results of acid effect

(Mean ± S.D. of three measurements)

<table>
<thead>
<tr>
<th></th>
<th>1h</th>
<th>2h</th>
<th>4h</th>
<th>8h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pure LIG</td>
<td>100±0.6</td>
<td>99.9±0.3</td>
<td>99.8±0.7</td>
<td>99.8±0.4</td>
</tr>
<tr>
<td>1%HCl</td>
<td>54.4±1.2</td>
<td>23.7±0.3</td>
<td>12.9±0.2</td>
<td>4.1±0.1</td>
</tr>
<tr>
<td>2%HCl</td>
<td>32.3±0.5</td>
<td>11.6±0.4</td>
<td>5.1±0.1</td>
<td>1.3±0.1</td>
</tr>
<tr>
<td>4%HCl</td>
<td>10.6±0.2</td>
<td>3.3±0.2</td>
<td>0.6±0.0</td>
<td>0.0±0.0</td>
</tr>
</tbody>
</table>

Figure 5-7. The results of the effect of acid. LIG all disappeared in 4% of HCl for 8hrs. The content of LIG was only 4.1% and 1.3% in 1%, 2% of HCl for 8h respectively. This indicated that LIG was hydrolyzed rapidly by a high concentration of acid. With the increase of time of LIG being in acid, some new peaks appeared and then disappeared on the HPLC chromatogram. This proved that acid had fatally influenced the LIG content. Thus LIG must be kept away from acid in the pharmaceutical preparation and application of LIG.
Table 5-7. The results of alkali effect

(Mean ± S.D. of three measurements)

<table>
<thead>
<tr>
<th></th>
<th>6h</th>
<th>12h</th>
<th>24h</th>
<th>48h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pure LIG</td>
<td>99.8±0.4</td>
<td>99.6±0.8</td>
<td>99.2±0.7</td>
<td>98.8±1.1</td>
</tr>
<tr>
<td>1%NaOH</td>
<td>96.9±0.6</td>
<td>93.1±1.0</td>
<td>91.5±0.8</td>
<td>90.8±1.2</td>
</tr>
<tr>
<td>2%NaOH</td>
<td>93.2±0.9</td>
<td>92.6±0.8</td>
<td>90.2±1.3</td>
<td>90.0±1.4</td>
</tr>
<tr>
<td>4%NaOH</td>
<td>91.4±0.5</td>
<td>90.5±1.4</td>
<td>89.2±1.5</td>
<td>87.3±1.1</td>
</tr>
</tbody>
</table>

Figure 5-8. The results of the effect of alkalinity. The content of LIG was not obviously decreased when NaOH was added into the LIG standard solution, and the content was 87.3% in 4% of NaOH for 48hrs. It indicated that the alkalinity had a little effect on the LIG stability.
Table 5-8. The results of metal ion effect

(Mean ± S.D. of three measurements)

<table>
<thead>
<tr>
<th></th>
<th>6h</th>
<th>12h</th>
<th>24h</th>
<th>48h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pure LIG</td>
<td>99.8±0.4</td>
<td>99.6±0.8</td>
<td>99.2±0.7</td>
<td>98.8±1.1</td>
</tr>
<tr>
<td>Fe³⁺</td>
<td>86.3±0.9</td>
<td>84.0±1.6</td>
<td>83.2±1.9</td>
<td>82.7±0.9</td>
</tr>
<tr>
<td>Cu²⁺</td>
<td>89.8±1.2</td>
<td>88.7±1.0</td>
<td>88.6±1.2</td>
<td>86.9±0.5</td>
</tr>
<tr>
<td>Fe²⁺</td>
<td>91.5±0.6</td>
<td>90.7±0.9</td>
<td>90.2±0.6</td>
<td>88.6±1.7</td>
</tr>
<tr>
<td>Zn²⁺</td>
<td>90.8±1.1</td>
<td>90.6±1.5</td>
<td>90.0±0.2</td>
<td>88.1±0.8</td>
</tr>
<tr>
<td>K⁺</td>
<td>91.1±1.6</td>
<td>90.2±0.4</td>
<td>90.0±0.7</td>
<td>87.7±1.2</td>
</tr>
<tr>
<td>Al³⁺</td>
<td>89.9±1.3</td>
<td>88.8±0.6</td>
<td>88.2±0.3</td>
<td>86.4±1.5</td>
</tr>
<tr>
<td>Na⁺</td>
<td>90.4±0.5</td>
<td>90.2±1.3</td>
<td>89.2±1.4</td>
<td>88.7±2.1</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>90.9±1.4</td>
<td>90.2±0.7</td>
<td>90.0±1.2</td>
<td>88.1±0.8</td>
</tr>
</tbody>
</table>

Figure 5-9. The results of the effect of metal ion. The content of LIG was a little decreased when metal ions solution was added into the LIG standard solution, and the lowest content of LIG was 82.7% in ferrous chloride after 48h. The results indicated that the common metal ions had no obviously effect on the LIG stability.
Table 5-9. The results of antioxidant effect

(Mean ± S.D. of three measurements)

<table>
<thead>
<tr>
<th></th>
<th>6h</th>
<th>12h</th>
<th>24h</th>
<th>48h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pure LIG</td>
<td>98.2±0.9</td>
<td>96.4±0.4</td>
<td>95.8±1.2</td>
<td>93.2±1.8</td>
</tr>
<tr>
<td>10% Vit E</td>
<td>98.8±1.2</td>
<td>97.6±1.3</td>
<td>96.8±0.4</td>
<td>95.9±1.2</td>
</tr>
<tr>
<td>5% Vit E</td>
<td>98.4±0.5</td>
<td>97.2±0.6</td>
<td>96.5±1.5</td>
<td>95.7±2.1</td>
</tr>
<tr>
<td>5% Vit C</td>
<td>94.9±0.9</td>
<td>92.1±0.7</td>
<td>85.3±1.9</td>
<td>68.6±1.4</td>
</tr>
<tr>
<td>10% Vit C</td>
<td>94.2±1.0</td>
<td>90.8±0.9</td>
<td>82.6±0.6</td>
<td>64.5±0.8</td>
</tr>
</tbody>
</table>

Figure 5-10. The results of the effect of antioxidant. The content of LIG was over 95% by heating at 50°C for 48hrs when Vitamin E was added into the LIG standard solution, it indicated that Vitamin E could protect LIG and improve the stability of LIG. The content of LIG was only about 65% by heating at 50°C for 48h when Vitamin C was added into the LIG standard solution, it indicated that Vitamin C for the acidic component could accelerate the LIG degradation because LIG was very unstable in the acidic condition.
CHAPTER 6

REFERENCE AND DEGRADATION STUDY OF LIGUSTILIDE

6.1 ABSTRACT

The stability of LIG was affected by various factors, and it was very difficult to maintain the stability of the high purity LIG. Based on the research to the factors affecting the LIG stability in Chapter 5, some suitable stabilizers were obtained to strengthen the stability of LIG, which included the No.1 and No.2 stabilizer. The stability effect of two stabilizers was compared in this experiment. It indicated No.2 stabilizer had better effect to LIG’s stability. Based on the investigations to the stabilizer, we finally developed the stable reference of LIG. The shelf life of LIG reference was predicted by accelerated testing, and it was 8 months with the LIG purity lowered by 2% at 25°C. The degradation products of LIG were further explored, and their structures were examined, also the degradation pathways of LIG were investigated. This provides a good basis for the new drug study of LIG.

6.2 INTRODUCTION

LIG, a very unstable compound, is an active ingredient of traditional herbal medicines
such as *Angelica sinensis*, *Ligusticum chuangxiong*, *Ligusticum wallichii*, and *Cnidium officinale*, etc. According to the paper (Li et al., 2000), pure LIG may degrade to 58% when it is conserved at 25°C for 15 days in daylight, and even if it is stored in the refrigerator at 4°C in darkness, it may degrade by nearly 15% in 15 days. It was reported that the stability of LIG in medical substances was much better because of the restriction by other phthalides in the herbs (Wang, 2003). *Angelica sinensis*, *Ligusticum chuangxiong*, *Ligusticum wallichii*, *Cnidium officinale*, etc., are all commonly used in China. There is a great market demand of the LIG reference for the quality control of these drugs. Due to the poor stability of LIG, currently there is no LIG reference on Chinese market, which has greatly restricted the research and development of these drugs. The instability causes much inconvenience to the preparation, storage and application of the reference of LIG. Based on the above investigations on the factors affecting the LIG stability, further systematic study was carried out to increase the stability of the LIG reference.

The purpose is to investigate the intrinsic stability of LIG and thus identify the likely degradation pathways as well as degradation products, which could provide scientific evidence for the manufacturing, package and storage of LIG, as well as for the establishment of a method to analyze its related substances. According to the investigation of the factors affecting the LIG stability in Chapter 5, LIG must be kept away from light and the influence of oxygen and temperature must be eliminated due to its own special chemical structure as well as the extremely poor stability. In the
package and storage of LIG, the influence of light and oxygen on the stability of the drug substance can be easily avoided by means of specific ways, but it is much more difficult to control the temperature factor. In storage the temperature can be kept very low, but during its manufacture and application, it is exposed to room temperature. Therefore it is a must to overcome the temperature influence for the LIG reference.

We had found out the stabilizer which could block the degradation of LIG with systematic experiments, but the shelf life of LIG in the stabilizer was unknown. The experimental periods limited the investigation of LIG’s long-term stability. Therefore, accelerated testing was performed to predict the LIG’s shelf life. Without knowing the relative parameters such as the orders and activation energy of the LIG degradation reaction, preliminary predictions for the shelf life of the LIG reference was carried out by using the initial average speed (IAS) method.

For innovate drugs, it is necessary to study the properties of their decomposition products. The determination of the degradation products and the related products plays an important role in the stability testing. While the study of the LIG reference was conducted, the degraded and isomerized production of LIG was also investigated by GC-MS. Supplementary information on the properties and the stability of LIG was obtained through such kind of research, which provided necessary data for the manufacturing, package and storage of LIG, thus provided benefit for further work on the pharmaceutical preparations and clinical application of LIG.
6.3 MATERIALS AND METHODS

6.3.1 Materials

6.3.1.1 Materials

Ethyl acetate was purchased from Beijing Chemical Co., Beijing, China. Methanol, hexane was from Dai Mo Chemical Co., Tianjin, China. Silica gel GF254 plate was from Ocean Chemical Ltd. Qingdao, China. HPLC grade methanol was purchased from Honeywell International Inc., Muskegon, USA and isopropyl alcohol was from Tedia Company, Fairfield, USA. Deionized water was generated from a Milli-Q water system (Millipore, Bedford, MA, USA).

6.3.1.2 LIG Preparation

LIG was extracted, separated and purified from Radix Angelica sinensis in our laboratory, with batch number: 050118. Purified LIG was identified by electron impact ionization (EI) MS, H\(^1\) NMR and C\(^{13}\) NMR spectrometric techniques. The purity was found to be > 98% based on the percentage of total peak area by GC assay.

6.3.2 METHODS
6.3.2.1 HPLC Assay of LIG

Please refer to 2.2.3.1.

6.3.2.2 LIG Reference Study

6.3.2.2.1 Selection of LIG Stabilizer

The LIG was taken and placed into the ampoules. After that, the tubes were filled with nitrogen to expel the oxygen. Then, the No.1 and No.2 stabilizer was added respectively into ampoules. The ampoules were individually sealed and then wrapped with tinfoil paper. Other LIG samples were prepared according to the above method but without the stabilizer. All ampoules containing LIG were heated in an oven at 60℃. The samples were taken out on 1, 2.5, 5, 7.5 and 10 day respectively, and the LIG content of each sample was determined by HPLC. The content variation was investigated and the suitable stabilizer was selected for LIG reference.

6.3.2.2.2 Preparation of LIG Reference

The high purity LIG was applied as the reference for LIG, and its purity was 99.85% based on the percentage of total peak area with GC assay. The optimum stabilizer
(No.2 stabilizer) was added to the ampoule and then filled with LIG. After that, the nitrogen was used to fill the ampoule for expelled the oxygen. The ampoule was sealed and wrapped with tin foil for the protection from light. A lot of LIG references were prepared.

6.3.2.3 Accelerated Testing for Shelf Life of LIG Reference

The initial average speed (IAS) was the average rate in the initial stage of degradation reaction of drug. To predict the drug’s shelf life, accelerated testing of the IAS was used with the content variation of drug in each unit time at the initial degradation stage. Providing reaction running at the temperature of \( T \), set the original drug concentration is \( C_0 \), and the drug concentration is \( C \) at period of \( t \), the IAS \( V_0 \) can be calculated by the following equation:

\[
V_0 = \frac{C_0 - C}{t}
\]

Providing different temperature and set as \( T_1, T_2, \ldots, T_i \) (i=8-9 usually), the experiment was conducted for i times, the corresponding IAS was obtained as \( V_{01}, V_{02}, \ldots, V_{0i} \), respectively. The following linear regression equation can be obtained from \( \log(V_0) \) versus \( 1/T \).

\[
\log(V_0) = \log(A) - \frac{E_a}{RT}
\]

This equation is quite similar to the Arrhenius Equation except for the replacement of \( k \) with \( V_0 \). According to this equation, the shelf life of the drug stored at room temperature can be predicted when \( T \) is refer to the room temperature. The purity
must be above 98% according to the requirement of chemical reference, and the shelf life of the LIG reference was calculated by using 2% of LIG degraded in accelerated testing.

The above prepared LIG reference was taken in a water bath with constant temperature of 50°C, 55°C, 60°C, 65°C, 70°C, 75°C, 80°C, 85°C respectively. The corresponding LIG reference in every temperature was taken out and determined the LIG content respectively according to the HPLC method. The time and the LIG content should be recorded when the LIG content was relatively decreased by about 2% under different temperature. According to the results, the linear regression equation of 1/T and lgV₀ was obtained. The shelf life of LIG reference in 25°C room temperature was predicted by the calculating equation of the IAS method.

6.3.2.4 Degradation Products Detection of LIG

The investigation on the properties of the LIG degradation products was needed, which could provide scientific evidence for manufacturing, package and storage of LIG. The high purity LIG sample was taken and placed into the ampoules but not sealed. These ampoules were influenced by light and oxygen, also placed at -20°C, 4°C and 25°C for 1 month, respectively. Then, these ampoules were taken out after 1 month. The appearance of each LIG sample was observed, and the TLC, HPLC and GC-MS were carried out in turn to investigate the degradation products of LIG.
6.3.2.4.1 Appearance Contrast

The appearance of high purity LIG reference was observed after the high purity LIG reference were placed at -20°C, 4°C and 25°C for 1 month respectively.

6.3.2.4.2 TLC Assay

10mg of pure LIG was taken and same amount of ASVO was taken. After that, the pure LIG sample was placed at 25°C for 1 month, then added 2ml of methanol to dissolve. In the TLC method, silica gel GF254 plate was used as the coating substance and hexane-ethyl acetate (10:1) as the mobile phase. The TLC method was carried out, the different LIG sample solution were taken and placed on the silica gel to develop.

6.3.2.4.3 HPLC Assay

10mg of the pure LIG samples, the LIG samples which kept at -20°C, 4°C and 25°C for 1 month and ASVO were taken into 10ml volumetric flask respectively. After that, the methanol was used to dissolved and diluted up to the volume of the volumetric flask. According to the above HPLC method, 10ul of different LIG sample solution was injected into the column, determined and recorded the chromatogram, and compared the peak and content of the main degradation products.
6.3.2.4.4 GC-MS Assay

10mg of LIG sample which kept at -20°C, 4°C and 25°C for 1 month were taken and 2ml of methanol was used to dissolve them respectively. The GC-MS method could refer to 2.2.1.4 and was carried out, the LIG sample solution was injected to the instrument and the result was examined. The total ion chromatogram and the mass spectrum of the main peak were recorded. The chemical structures of the main degradation products were analyzed and validated.

6.4 RESULTS

6.4.1 LIG Reference Study

6.4.1.1 Selection of LIG Stabilizer (Table 6-1, Fig 6-1)

According to the preparation method of LIG reference, three types of LIG samples were prepared which were contained No.1 stabilizer, No.2 stabilizer and no stabilizer respectively. The above samples were heated in an oven at 60°C. The samples were taken out on Days 1, 2.5, 5, 7.5 and 10 respectively, and the LIG content of each sample was determined by HPLC. The results are presented in Table 6-1 and Fig 6-1. The results show that the stabilizers had obvious potent protective effect on LIG. The
content of LIG in the No.2 stabilizer was only decreased to 50% approximately at 60°C after 10 days. The No.2 stabilizer had a much better protective effect than that of No.1, which even could be 10 times as those which have no stabilizer incorporated. The content of LIG with no stabilizer incorporated decreased rapidly at 60°C without any influence from oxygen or light, and only 4.7% of LIG remained at Day 10. No.2 stabilizer was effective for the protection of LIG and the retarding of its degradation reaction. Therefore, No.2 stabilizer was chosen as the stabilizer of LIG reference in the following experiments.

6.4.1.2 Preparation of LIG Reference (Fig 6-2)

The LIG purity was 99.85% as confirmed by GC assay, and No.2 stabilizer was added to LIG. The oxygen in the ampoule was expelled by nitrogen. The naked sample was indicated in Fig 6-2. Then they were sealed and wrapped with tin foil to keep away from light, and stored in a -20°C or 4°C refrigerator.

6.4.2 Accelerated Testing for Shelf Life of LIG Reference (Table 6-2, Fig 6-3)

The above prepared LIG reference was taken into a water bath with constant temperatures of 50°C, 55°C, 60°C, 65°C, 70°C, 75°C, 80°C, 85°C respectively. The corresponding LIG reference in every temperature was taken out and the content determined by HPLC. When the LIG content was decreased about 2% under different
temperature, the time and LG content were recorded respectively.

The results are shown in Table 6-2 and Fig 6-3. Base on the value of $1/T$ on X-axis and $\log V_0$ on Y-axis, the equation of linear regression $\log V_0 = 15.445 - 5634/T$ ($r=0.9956$) was obtained, and the correlation coefficients ($r$) were greater than 0.995, signifying the linear relation between $1/T$ and $\log V_0$. Therefore, at 25°C, the time for the content of LIG reference dropped from the marked content 100% to 98% could be calculated by the equation as: $t = 5782h$ (about 8 months), which means at room temperature of 25°C, the prepared LIG reference can be kept stable for 8 months. It indicated that the prepared LIG reference could be kept at room temperature for over half a year, which prolonged greatly the stable shelf life of LIG. It was just an approximate prediction of the shelf life of LIG reference by accelerated testing. However, the temperature is still a comparative evident influential factor for the LIG stability. Due to the extreme instability of LIG, the storage for LIG reference in a hypothermic refrigerator is still recommended, for a longer shelf life will be obtained in this way.

6.4.3 Degradation Products Detection of LIG

6.4.3.1 Appearance Contrast (Fig 6-4)

The appearance of the high purity LIG which kept at -20°C, 4°C and 25°C for 1
month was observed. The result is in Fig 6-4. With the increase of temperature, the slightly yellowish oily liquid of LIG was changed to a brown red liquid substance.

6.4.3.2 TLC Assay (Fig 6-5)

The TLC method was carried out by using the sample solution of pure LIG, ASVO and the LIG samples which placed at 25°C for 1 month respectively. After that, the LIG samples were placed in the silica gel plate to develop and examined. Then, the position and color of the spots were recorded on the chromatogram and were compared with each other. The result is in Fig 6-5. It indicated that the LIG sample which placed at 25°C for 1 month had many degradation produces was appeared, and some degradation products were similar to the compounds of ASVO.

6.4.3.3 HPLC Assay (Fig 6-6, 6-7, 6-8, 6-9)

The HPLC method was carried out, 10ul of different solution of the pure LIG, the LIG at -20°C, 4°C and 25°C for 1 month and ASVO was were injected to the instrument respectively. The chromatogram was determined and recorded respectively (Fig 6-6, 6-7, 6-8, 6-9), and the content of LIG was calculated by contrasting the pure LIG. From Figs 6-6, 6-7, 6-8, 6-9, the retention time of LIG was 9.9 minute. The LIG content was 98.2%, 72.4% and 15.7% at -20°C, 4°C and 25°C for 1 month respectively. LIG was more degraded with an increase of the environment
temperature. Some degradation products were similar to the compounds of *Angelica*

### 6.4.3.4 GC-MS Assay (form Fig 6-10 to Fig 6-22)

The GC-MS method was carried out, 0.5ul of the different sample solution of the LIG at -20°C, 4°C and 25°C for 1 month were injected into the instrument respectively. After that, the total ion chromatogram and the mass spectrum of each peak was determined and recorded. The main degradation compounds were recorded and validated. The results which are shown in Fig 6-10 to Fig 6-21 indicated that the degradation products appeared more and more with the increasing storage temperature under the influence of light and oxygen. The validated chemical formulas of the degradation products included C₈H₄O₃, C₈H₆O₃, C₁₂H₁₂O₂, C₁₂H₁₂O₂, C₁₂H₁₄O₂, C₁₂H₁₄O₄, C₁₂H₁₄O₃, C₁₆H₂₄O₄ and so on. The chemical structures are shown in Fig 6-13 to Fig 6-21. According to the structure of the degradation compounds, the basic degradation pathway of LIG was deducted, and showed in Fig 6-22. However, the degradation reaction of LIG was a complicated multiple reaction and there might be other degradation pathways.

### 6.5 DISCUSSION

Temperature, oxygen and light have great influence on the stability of LIG. For light and oxygen, it is easy to eliminate their influence. But for temperature, it acts as the
most difficult factor to get over with. In this study, in order to get a suitable stabilizer to improve the chemical stability of LIG, factors that may affect its stability were investigated. To prepare the LIG reference, there were specific requirements for the added stabilizer. It must have no influence on the quality of LIG and no inlet of other ectogenic substances. And the stabilizer must have a great improvement on the stability of LIG in severe conditions, which are also critical to the successful development of LIG reference. Finally suitable stabilizer for LIG was provided. Also it was proved to have an obvious protective effect on LIG against high temperature.

In the study on the shelf life of LIG reference, since the LIG stability is affected by the factors of oxygen, temperature, light etc., the degradation reaction mechanisms must be different at different times and temperatures, thus leading to different reaction order and activation energy. Accordingly, we applied the IAS method, which was not influenced by reaction order or activation energy, to predict the shelf life preliminarily. This method has been proved to be convenient and fast with a better accuracy. Also the elementary study was carried out on the prepared LIG reference with the accelerated testing. Then the shelf life of the LIG purity was found to be decreased by 2% at room temperature of 25°C after 8 months, indicating that the prepared LIG reference had a good stability and could meet the required standard. The temperature, however, still tended to be a comparative evident influential factor to LIG’s stability. For normal storage, the replacement of LIG in a hypothermic refrigerator is still recommended, as a longer shelf life could be obtained in this way.
The degradation reaction of LIG is a complicated multiple reaction. Oxidation, hydrolysis and photo-degradation are the major degradation routes of LIG. The degradation reaction mechanism is different in different storage conditions. For innovating a new drug, it is necessary to study the properties of their degradation products. The determination of degradation products and the related products plays an important role in the research and development of a new drug. In this experiment, investigations applying GC-MS were also performed on the degradation productions of LIG, and the structure of the degradation compounds was obtained. Then the basic degradation pathway of LIG was deducted in Fig 6-22. It would benefit further work on the pharmaceutical preparations and clinical application of LIG.
Table 6-1. The results of LIG stabilizer selection.

(Means ± S.D. of three measurements)

<table>
<thead>
<tr>
<th></th>
<th>1d</th>
<th>2.5d</th>
<th>5d</th>
<th>7.5d</th>
<th>10d</th>
</tr>
</thead>
<tbody>
<tr>
<td>no stabilizer</td>
<td>69.2 ± 1.4</td>
<td>48.3 ± 0.9</td>
<td>23.6 ± 1.3</td>
<td>8.8 ± 0.9</td>
<td>4.7 ± 0.6</td>
</tr>
<tr>
<td>No.1 stabilizer</td>
<td>79.1 ± 2.0</td>
<td>65.2 ± 1.4</td>
<td>48.9 ± 2.1</td>
<td>38.6 ± 2.3</td>
<td>28.4 ± 1.3</td>
</tr>
<tr>
<td>No.2 stabilizer</td>
<td>87.2 ± 2.1</td>
<td>74.5 ± 1.6</td>
<td>61.7 ± 1.9</td>
<td>52.1 ± 1.3</td>
<td>45.1 ± 2.2</td>
</tr>
</tbody>
</table>

Figure 6-1. The results of LIG stabilizer selection. The content of LIG with no stabilizer incorporated decreased rapidly at 60°C without oxygen and light influence, and only 4.7% of LIG remained at Day 10. The stabilizer had obvious potent protective effect for LIG. The No.2 stabilizer had a much better protective effect than the No.1 stabilizer, with which the content of LIG decreased by 50% approximately at 60°C after 10 days, about 10 times higher than the one with no stabilizer incorporated. No.2 stabilizer was effective for the protection of LIG and the retarding of its degradation reaction. Therefore, No.2 stabilizer was chosen as the stabilizer of LIG reference.
Figure 6-2. The samples of LIG reference. No.2 stabilizer was added to LIG ampoule being expelled from oxygen by nitrogen. It was naked sample without tinfoil paper.

Table 6-2. The results of accelerated testing for shelf life of LIG reference

*(Mean ± S.D. of three measurements)*

<table>
<thead>
<tr>
<th>T(℃)</th>
<th>1/T(10⁻³)</th>
<th>t(h)</th>
<th>C(%)</th>
<th>C0—C(%)</th>
<th>V₀</th>
<th>1gV₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>55</td>
<td>3.05</td>
<td>98</td>
<td>98.04±0.62</td>
<td>1.96</td>
<td>0.020</td>
<td>−1.699</td>
</tr>
<tr>
<td>60</td>
<td>3.00</td>
<td>60</td>
<td>97.95±0.73</td>
<td>2.05</td>
<td>0.034</td>
<td>−1.466</td>
</tr>
<tr>
<td>65</td>
<td>2.96</td>
<td>30</td>
<td>98.08±0.95</td>
<td>1.92</td>
<td>0.064</td>
<td>−1.194</td>
</tr>
<tr>
<td>70</td>
<td>2.91</td>
<td>24</td>
<td>97.84±0.68</td>
<td>2.16</td>
<td>0.090</td>
<td>−1.046</td>
</tr>
<tr>
<td>75</td>
<td>2.87</td>
<td>12</td>
<td>98.02±0.93</td>
<td>1.98</td>
<td>0.165</td>
<td>−0.783</td>
</tr>
<tr>
<td>80</td>
<td>2.83</td>
<td>6</td>
<td>97.93±0.66</td>
<td>2.07</td>
<td>0.345</td>
<td>−0.462</td>
</tr>
<tr>
<td>85</td>
<td>2.79</td>
<td>3</td>
<td>98.14±0.41</td>
<td>1.86</td>
<td>0.620</td>
<td>−0.208</td>
</tr>
<tr>
<td>90</td>
<td>2.75</td>
<td>2</td>
<td>98.26±0.57</td>
<td>1.74</td>
<td>0.870</td>
<td>−0.060</td>
</tr>
</tbody>
</table>
Figure 6-3. The results of accelerated testing for shelf life of LIG reference. With the value of $1/T$ on X-axis and $\lg V_0$ on Y-axis, the equation of linear regression was obtained as: $\lg V_0 = 15.445 - 5634/T$ (r=0.9956), and the correlation coefficients (r) were greater than 0.995, signifying the linear relation between $1/T$ and $\lg V_0$. Therefore, at 25°C, the time period during which the content of LIG reference dropped from the marked content of 100% to 98% could be calculated by the equation as: $t = 5782$ h (about 8 months), which means at room temperature of 25°C, the prepared LIG reference can be kept stable for 8 months.

Figure 6-4. The appearance of LIG at -20°C, 4°C and 25°C after 1 month (left: -20°C; middle: 4°C; right: 25°C). With the increasing of the temperature, the slightly yellow oily liquid LIG was changed to the brown red liquid substance.
Figure 6-5. The TLC chromatogram of degradation products TLC testing of LIG (1: LIG reference, 2: LIG sample at 25°C for 1 month, 3: ASVO. left: ultraviolet light 254nm, middle: ultraviolet light 365nm, right: dyed with iodine vapor). It indicated that the many degradation products of LIG appeared in the LIG sample at 25°C for 1 month, and some degradation products were similar to the compounds of ASVO.
Figure 6-6. The HPLC chromatogram of LIG sample at -20°C for 1 month. The retention time of LIG was 9.9 minute, and the measured content of LIG was 98.2%.

Figure 6-7. The HPLC chromatogram of LIG sample at -4°C for 1 month. The retention time of LIG was 9.9 minute, and the measured content of LIG was 72.4%.
Figure 6-8. The HPLC chromatogram of LIG sample at 25°C for 1 month. The retention time of LIG was 9.9 minute, and the measured content of LIG was 7%.

Figure 6-9. The HPLC chromatogram of *Angelica sinensis*. The retention time of LIG was 9.9 minute.
**Figure 6-10.** The total ion chromatogram of the LIG at -20°C for 1 month. The degradation product of LIG did not appear. The main peak was the LIG. And the retention time of LIG was 8.4 min.

**Figure 6-11.** The total ion chromatogram of the LIG at 4°C for 1 month. The some degradation products of LIG appeared.
Figure 6-12. The total ion chromatogram of the LIG at 25°C for 1 month. The many degradation products of LIG appeared.
Figure 6-13. The mass spectrum of the compound that the retention time is 3.6 min.

The molecular formula is $\text{C}_8\text{H}_4\text{O}_3$ with molecular weight of 148, and the chemical structure is the follow as:
Figure 6-14. The mass spectrum of the compound that the retention time is 3.9 min.

The molecular formula is C₈H₆O₃ with molecular weight of 150, and the chemical structure is the follow as:
Figure 6-15. The mass spectrum of the compound that the retention time is 7.6 min.

The molecular formula is $C_{12}H_{12}O_2$ with molecular weight of 188, and the chemical structure is the follow as:
Figure 6-16. The mass spectrum of the compound that the retention time is 8.2 min. The molecular formula is $\text{C}_{12}\text{H}_{12}\text{O}_{2}$ with molecular weight of 188, and the chemical structure is the follow as:

\begin{center}
\includegraphics[width=0.5\textwidth]{chemical_structure.png}
\end{center}
Figure 6-17. The mass spectrum of the compound that the retention time is 8.4 min.

The molecular formula is C_{12}H_{14}O_{2} with molecular weight of 190, and the chemical structure is the follow as:
**Figure 6-18.** The mass spectrum of the compound that the retention time is 9.2 min.

The molecular formula is $\text{C}_{12}\text{H}_{14}\text{O}_2$ with molecular weight of 190, and the chemical structure is the follow as:
Figure 6-19. The mass spectrum of the compound that the retention time is 9.5 min.

The molecular formula is $C_{12}H_{14}O_4$ with molecular weight of 222, and the chemical structure is the follow as:
**Figure 6-20.** The mass spectrum of the compound that the retention time is 10.6 min.

The molecular formula is $\text{C}_{12}\text{H}_{14}\text{O}_3$ with molecular weight of 206, and the chemical structure is the follow as:
Figure 6-21. The mass spectrum of the compound that the retention time is 12.6 min.

The molecular formula is \( \text{C}_{16}\text{H}_{24}\text{O}_4 \) with molecular weight of 280, and the chemical structure is the follow as:
Figure 6-22. The basic degradation pathway of LIG. According to the structure of the degradation compounds, the basic degradation pathway of LIG was deducted. However, the degradation of LIG was very complex and had maybe other different pathways.
CHAPTER 7

SYNTHESIS OF LIGUSTILIDE

7.1 ABSTRACT

Synthesis of LIG was studied in our experiment by using phthalic anhydride as raw material: firstly 3-butylidene-phthalide needed to be obtained from the reaction between phthalic anhydride and butyl lithium; and then LIG was synthesized from 3-butylidene-phthalide through Birch reaction. However, this synthesis had no industrialized productive value, as the yield rate of LIG was too low, yet the synthesis condition was pretty strict. From this aspect, we still had a very long way to go to increase its product yield rate for industrial production.

7.2 INTRODUCTION

The LIG content in traditional Chinese medicine is very high, and the output of its raw material is suitable and could satisfy the need of industrialization production. But the nature couldn’t always offer resources without limit, no matter in the now or future. The synthesis of LIG has also drawn more attention. Li et al. reported a detailed synthesis of LIG with four steps (Li et al., 1994). In this synthesis, the preparation of LIG comprised the reaction between o-carboxybenzaldehyde and
KOCMe₃ with butyltriphenyolphosphonium bromide to give a cis-trans mixture of o-(1-penten-1-yl) benzoic acid, the oxidation with peracetic acid to give a mixture of threeo- and erythro-3-(1-hydroxybutyl) phthalide, the reduction with Na-NH₃ to give a mixture of threeo- and erythro-4,5-dihydro -3-(1-hydroxybutyl)phthalide, and the subsequent reaction at room temperature to give LIG. But the synthesis yield rate of LIG was only about 5% (Li et al., 1994). It was also reported that the synthesis of naturally occurred LIG was accomplished by the cyclization of o-1-pentynylbenzoic acid, which was prepared from 1,4-cyclohexanedione monoethylene ketal in several steps, and then catalyzed by silver iodide or silver in a mild reaction condition as a key step (Ogawa et al., 1995). The synthesis yield rate was low as well. Also the synthesis process of LIG was complicated, and the instable problem of LIG had great influence to the product yield. In one word, it still had a long way to go to enhance the LIG product yield and thus industrialize its production.

Also, for LIG, it was reported that it had ever been synthesized successfully (Li et al., 1993), but the synthesis just had a very low yield with a large number of steps. Meanwhile, LIG and 3-butylidene-phthalide, two main active components of Angelica sinensis volatile oil (ASVO), were phthalide derivatives that had very similar structure. Also LIG had one double bond less than 3-butylidene-phthalide in 6-membered ring. So once 3-butylidene-phthalide was synthesized, LIG could be accordingly synthesized from it with one step only. This idea of synthesis was ever reported but no experiment was fulfilled to support it (Li et al., 1995). Anyway, it was
really a good idea and assumed a good synthesis way for LIG, as it’s much easier to synthesize 3-butylidene-phthalide first and then into LIG. There were only 2 steps in all for LIG synthesis using this way. Therefore, our experiment mainly focused on the study of this good synthesis way.

7.3 MATERIALS AND METHODS

7.3.1 Materials

7.3.1.1 Materials

Phthalic anhydride and methyl toluenesulfonate (P-TsOH) were purchased from Guangzou Chemical Co., Guangzhou, China. Butyl Lithium was purchased from Ganfeng Lithium Co., Ltd in Jiangxi Province, China. Tetrahydrofuran (THF), benzene, methanol, Na$_2$SO$_4$, HCl, NaCl, NaHCO$_3$ and hexane were from Dai Mo Chemical Co., Tianjin, China. Silica gel HF254 plate was from Ocean Chemical Ltd. Qingdao, China. Deionized water was generated from Milli-Q water system (Millipore, Bedford, MA, USA).

7.3.1.2 Reagent Preparation

Preparation of Phthalic Anhydride
Phthalic anhydride was put into a drier to dry for 72 hours. Then it was heated to sublime and then sublimation which was the pure phthalic anhydride was collected for use.

**Preparation of Anhydrous THF**

NaH was added into THF and marinated for 48 hours. Then natrium and dibenzophenone were added for heating until it turned into dark blue colour. After that, anhydrous THF was steamed out from the mixture for use.

**Preparation of Alass Apparatus**

All glass apparatus were heated at 110°C for 24 hours until they got dried. Then they were cooled down for use.

**7.3.2 METHODS**

**7.3.2.1 Synthesis of 3-Hydroxy-3-butylphthalide**

Please refer to 2.2.7.1.

**7.3.2.2 Synthesis of 3-butylidene-phthalide**

Please refer to 2.2.7.2.
7.3.2.3 Synthesis of LIG

Please refer to 2.2.7.3.

7.4 RESULTS

7.4.1 Synthesis of 3-Hydroxy-3-butylphthalide

Without any water and oxygen, 7.423g of phthalic anhydride was dissolved into anhydrous THF. 20ml of 20% butyl lithium was dropped into it with the protection of argon. After stirring for 45 minutes at room temperature, 30ml of water was added and then THF was removed through decompression. The leftover was acidified by HCl and extracted by ether. Then the extraction was washed by saturated NaCl solution and dried by anhydrous Na$_2$SO$_4$ for 24 hours. At last, the mixture was concentrated and the yellow oily substance was got.

7.4.2 Synthesis of 3-butylidene-phthalide (from Fig 7-2 to Fig 7-6)

The above oily substance was dissolved into 60ml of benzene, and a little amount of P-T$_3$OH was added to it. The mixture was stirred for 2 hours with heating. Then it was dried by anhydrous Na$_2$SO$_4$ after washed by saturated NaCl solution. A yellowish oily
substance was obtained after the solvent was removed by decompression. There was 3.6% of 3-butylidene-phthalide in this substance which via GC-MS detection (Fig 7-2, 7-3). Then 0.31g of compound was obtained after separation by way of silica gel column chromatography. This compound was confirmed as 3-butylidene-phthalide (Fig 7-4) with structure assessment by GC-MS (Fig 7-3) and NMR (Fig 7-5, 7-6).

7.4.3 Synthesis of LIG (from Fig 7-7 to Fig 7-10)

0.3g of 3-butylidene-phthalide was dissolved into 10ml of anhydrous THF. After that, 0.5g of 2-butyl alcohol, 50mL of fluid ammonia as well as 0.05g of Lithium was added into at -60°C. Then it was stirred for 6 hours. During that period, the mixture turned blue gradually. 3g of ammonium chloride was added at the same temperature till the blue faded away. After another 2 hours of stirring, it was put into room temperature. Then ammonia was volatilized thoroughly. And the leftover was melted by 50g of ice and acidified by HCl till to pH about 5 to 6. After it was extracted by ether and then dried by anhydrous Sodium Sulfate, the mixture was filtrated. Also the ether was steamed away by decompression. Then the mixture became a red oily substance weighing at 0.15g. After the separation, by way of silica gel column chromatography, the leftover turned into a yellow oily substance weighing at only 0.01g. This compound was confirmed as LIG (Fig 7-7) from structure assessment by GC-MS (Fig 7-8) and NMR (Fig 7-9, 7-10). The yield rate of synthesis LIG from 3-butylidene-phthalide was very low at only about 3%.
7.5 DISCUSSION

In the synthesis of 3-butylidene-phthalide, we ever tried several materials as solvent, and finally got a conclusion that, only THF was available to make 3-butylidene-phthalide. Also, for the reaction temperature, we found that, only at 25°C could 3-butylidene-phthalide be obtained. Moreover, when it was at 0°C or even lower, no output could be made. And when it was at 60°C, most of the output became a substance with molecular weight at 246. This substance was confirmed as 3,3-dibutyl-phthalide (Fig 7-11) with structure assessment by NMR (Fig 7-12, 7-13). Also, from its structure we learned that, two butyls were brought into the third position of phthalic anhydride. That’s because the reaction temperature was so high that it had strongly enhanced the activity of butyl lithium, thus brought in two butyls. Meanwhile, if the temperature was too low, no butyl could be brought in at all. Therefore, the reaction temperature should be under well control as a key point.

The key step in LIG synthesis is the Birch reaction of 3-butylidene-phthalide. In this experiment, phthalic anhydride was used as raw material, and then butyl from butyl lithium was adopted as introductive functional group. 3-butylidene-phthalide was finally obtained through the reaction after P-TsOH and Benzene was added. It was ever reported (Li et al., 1995) before, however, the report, in which Natrium was used as reactant, didn’t have any LIG made out till the very end. Now in this experiment,
after several trial and error, finally Lithium was selected to replace Natrium and then LIG was obtained successfully. Even though, the yield rate was still very low at only about 3%, but the condition of synthesis was pretty critical, which have brought great inconvenience to its industrialization of manufacture.

In this experiment, phthalic anhydride was used as raw material, and then butyl from butyl lithium was adopted as introductive functional group. 3-butylidene-phthalide was finally obtained through the reaction after P-TsOH and Benzene was added. It had the advantages for fast reaction, high yield and easy separation, while the disadvantage was critical reacting condition which required preparation without any water and oxygen, also the activity of organic metal reagent was a bit high and hazardous to control. We had ever tried many ways of synthesis, but only failed to find a better way to make LIG more easily with much higher yield rate. Moreover, LIG itself still had very special chemical structure as well as high instability, which had made its synthesis become difficult. Thus, the industrialization of manufacturing LIG seemed to be unpractical at present.
Figure 7-1. The synthesis pathway of LIG.
**Figure 7-2.** The total ion chromatogram of the 3-butyldiene-phthalide synthesis substances in GC-MS. There was the peak of 3-butyldiene-phthalide at 11.5min, and the content of 3-butyldiene-phthalide was 3.6% in the total synthesis substance which via GC-MS detection.

**Figure 7-3.** The mass spectrum of the 3-butyldiene-phthalide
Figure 7-4. The structure of 3-butyldiene-phthalide
Figure 7-5. The $^1$H—NMR spectrum of the synthesized 3-butylidene-phthalide.

Figure 7-6. The $^{13}$C-NMR spectrum of the synthesized 3-butylidene-phthalide.
Figure 7-7. The structure of the synthesized LIG

Figure 7-8. The mass spectrum of the synthesized LIG.
Fig 7-9. The $^1$H-NMR spectrum of the synthesized LIG

Figure 7-10. The $^{13}$C-NMR spectrum of the synthesized LIG
**Figure 7-11.** The chemical structure of 3,3-dibutyl-phthalide

**Figure 7-12.** The $1H$–NMR spectrum of the synthesized 3,3-dibutyl-phthalide.
Fig 7-13. The $^{13}$C-NMR spectrum of the synthesized 3-butylidene-phthalide.
CHAPTER 8

CYCLODEXTRIN PREPARATION OF LIGUSTILIDE

8.1 ABSTRACT

The cyclodextrin (CD) inclusion of LIG in powdery form is quite important to improve its stability and solubility, as well as to expand its application. Hydroxylpropyl-β-cyclodextrin (HP-β-CD) was adopted as the host material for LIG inclusion complex. The inclusion process with HP-β-CD was optimized in this experiment. The stability, solubility and inclusion degree of LIG CD inclusion complex were determined. The results indicated that the stability and aqueous solubility of LIG were improved by the CD inclusion complex. Therefore, the LIG CD inclusion complex was capable of serving for the pharmaceutical preparations for oral administration and injection.

8.2 INTRODUCTION

The CD inclusion complexes have many advantages for drugs, which include improving stability, increasing solubility and bioavailability, reducing toxicity and side effects, eliminating abnormal taste and thrilling odor, transforming liquid drugs
into solid state and so on (Archontaki et al., 2002). The stability of many drugs is also affected by the environment such as temperature, humidity, light and oxygen in air (Buvari and Barcza, 1999). With the application of CD inclusion, the drug is contained in a molecule capsule, thus being isolated from the surroundings and could maintain a stable state. In this way, the drugs not only gain a much better solubility, but also possess a greatly enhanced stability as well as the abilities of anti-oxygen, anti-light, and thermo-resistance (Xiang and Anderson, 2002). Inclusion complexes can increase the aqueous solubility of drugs and increase the bio-availability. The drug level in blood will be increased correspondingly after administration, which is beneficial to the drug absorption in vivo and improving bioavailability and solubility of the drugs. Moreover, the permeability of cell membrane is promoted to favor the absorption of drugs. By the inclusion with CD, the drugs’ side effects as well as stimulation to the skin, laryngeal, intestines and stomach can be reduced. The bitter, acerbity and abnormal taste are the problems often encountered in the formula design of drugs. The taste can be masked when a CD exists and forms inclusion complexes with the drug molecules. After the liquid drug gets into the molecule cavity of CD, the liquid drug can be dried together with CD and transformed to powder.

HP-β-CD is one of the derivatives of β-CD, which consists of 7 chair-conformation glucose molecules (Kawasaki et al., 2001). β-CD is a bucket-shaped molecule with openings at both ends and the upper part is wider than the lower part as indicated in Fig 8-1. The 3-D structure of β-CD is shown in Fig 8-2. The wider opening side
consists of 14 para-hydroxyl and the narrow opening side consists of 7 primary hydroxyl. So the outer surface of β-CD is hydrophilic in nature. The surface of the lumen consists of C-H bond and ether bond, so the lumen surface is hydrophobic in nature. Hydrophobic molecules with suitable size can be contained in the lumen to form an inclusion complex. Due to its special molecular structure, CD has an important characteristic of taking the guest molecule into the hydrophobic lumen of CD, which leads to changes of the physical and chemical properties of the guest molecule. In recent years, the properties of β-CD have been improved by changing the functional groups as shown in Fig 8-3. The aqueous solubility of HP-β-CD increases while toxicity and incentive decrease.

Drug molecules can be included in CD in 2 major forms, which are 1:1 or 1:2 as guest (drug) to host (CD) molecule ratio, as shown in Fig 8-4 and Fig 8-5. The formation of inclusion complexes, which is a physical process, does not involve any chemical reaction or formation of ionic and covalent bond (Yoshii et al., 2006). The formation of inclusion complexes depends on the polarity of the host and guest molecules. The stability of the inclusion complexes is determined by the strength of the van der Waals’ force between the host and guest molecules. If the size of the guest molecule is too small, the van der Waals’ force is small and the guest molecule can get in and out of the lumen of CD freely. If the size of the guest molecule is too large, the guest molecule is difficult to enter into the lumen of CD or just have the side chain get inside the lumen (Zhu et al., 1996). Both of the above cases cannot form stable
inclusion complexes. Stable inclusion complexes can only be formed when the size of the guest molecule matches with the size of the lumen of CD.

There are many methods to prepare inclusion complexes (Ikeda et al., 2002). In practical study and application, a proper method is selected depending on the properties of both the host and guest molecules, as well as the proportion of the incorporated formula ingredients. Commonly used methods include saturation of aqueous water, ultrasonic concussion, stirring method, trituration method, freeze-drying, spray drying, etc., which should be chosen according to the properties of the drug. Besides, the formation of inclusion complexes is affected by extrinsic conditions such as reaction time, reaction temperature, the time for stirring (or ultrasonic concussion), and the concentration of the reactant. Generally, the formation of inclusion complexes may profit from a longer reaction time and stirring time, as well as an enhanced concentration of CD.

As a result of its strong hydrophilicity, CD is unable to permeate through the lipophilic biomembrane to enter blood circulation (Shehatta et al., 2005). The absorption mechanism of drugs will not be changed after the administration of certain kind of pharmaceutical preparation of the inclusion complexes. Therefore, CD is an ideal carrier for liposoluble and unstable drugs. LIG is a liposoluble component with a comparatively low polarity and poor stability, a simple molecular structure and a lower molecular weight. This kind of substance, with a small molecule and low
polarity, is easily included by CD. In view of the above described advantages of CD, the inclusion of LIG with CD is an important measure to ameliorate the physical chemical properties of LIG.

CD attracts more and more attention in pharmaceutical research due to its superiority for preparation of drugs. Among all kinds of CD, HP-β-CD, which is known for having the least toxicity and side effect in the pharmaceutical industry, is the most widely used in clinic applications and has the best prospect (Gould and Scott, 2005). It is the first excipient of CD for intravenous injection approved by FDA, and was recorded as an excipient for injection in the U.S. Pharmacopeia as well as in the Handbook of Pharmaceutical Excipients by Wade A. Currently, HP-β-CD is widely used in the pharmaceutical preparation of drugs for oral administration, injection, transdermal drug delivery, nasal drug delivery and rectal drug delivery. Therefore, HP-β-CD was chosen as the host substance of inclusion for the study on LIG inclusion complexes in this experiment.

8.3 MATERIALS AND METHODS

8.3.1 Materials

8.3.1.1 Materials
Hydroxylpropyl-β-Cyclodextrin (HP-β-CD) was purchased from BASF Chemical Co., German. Ethyl acetate was from Beijing Chemical Co., Beijing, China. Methanol, ethanol, hexane were from Dai Mo Chemical Co., Tianjin, China. Silica gel GF254 plate was from Ocean Chemical Ltd. Qingdao, China. HPLC grade methanol was from Honeywell International Inc., Muskegon, USA and isopropyl alcohol was from Tedia Company, Fairfield, USA. Deionized water was generated from a Milli-Q water system (Millipore, Bedford, MA, USA).

8.3.1.2 LIG Preparation

LIG was extracted, separated and purified from Radix *Angelica sinensis* in our laboratory, with the batch number: 050118. Purified LIG was identified by electron impact ionization (EI) MS, H\(^1\) NMR and C\(^{13}\) NMR spectrometric techniques. The purity was found to be > 98% based on the percentage of total peak area by GC analysis.

8.3.2 METHODS

8.3.2.1 Preparation Process Optimization of LIG CD Inclusion Solution

Accurately weighed 10g of HP-β-CD and dissolved in 10ml deionized water at 50°C in a conical flask with a magnetic bar providing a stirring force to enhance the
dissolution of HP-β-CD, to ensure all HP-β-CD was dissolved. Then the saturated HP-β-CD solution was allowed to cool down to room temperature. Dissolved 1g of LIG in 1ml ethanol, and then added it into the above saturated HP-β-CD solution. Then the following single factor tests were done to optimize the best preparation process by comparing the efficiency of different preparations on the LIG-CD inclusion complexes using the physical stability of LIG-CD solutions as the index.

8.3.2.1.1 Selection of Preparation Methods

There are different methods for the preparation of inclusion complexes. In order to determine which method gives the best efficiency, we should try different methods to prepare the inclusion complexes of LIG with HP-β-CD.

To find out the best method for the preparation of LIG-CD inclusion complexes, three different methods, magnetic stirring, ultrasonic shaking and high speed stirring, were chosen to prepare the LIG-CD inclusion complex, respectively.

- **Magnetic stirring:** The above mixed solution of 10g HP-β-CD and 1g LIG was stirred by a magnetic bar for 30min at room temperature.

- **Ultrasonic shaking:** Ultrasonic, instead of stirring, provided the mixing force. The above mixed solution of 10g HP-β-CD and 1g LIG was sonicated for 30min.

- **High speed stirring:** The above mixed solution of 10g HP-β-CD and 1g LIG was stirred by a homogenizer. The stirring speed of the homogenizer was 20000 r/min.
The time of stirring by the homogenizer was 3min.

The LIG-CD inclusion solutions prepared with the above three methods were placed and observed at room temperature to compare their efficiency. The LIG-CD inclusion solutions prepared by magnetic stirring and ultrasonic shaking began to delaminate obviously after one day, and the LIG was presented at the surface of the solution clearly after three days, which indicated that LIG had not formed complex with CD. This indicated that the inclusion capacity of the CD prepared by these two methods was weak. However, the LIG-CD inclusion solutions prepared by high speed stirring was still homogeneous, neither delaminated nor with freed LIG, which indicated that the inclusion capacity prepared by this method was strong. Finally, high speed stirring was chosen as the method to prepare the LIG-CD inclusion complexes.

8.3.2.1.2 Selection of Stirring Speed

The above mixed solutions of 10g HP-β-CD and 1g LIG were stirred by a homogenizer. The stirring speed of the homogenizer was 10000 r/min, 15000 r/min, 20000 r/min, 25000 r/min, 30000 r/min and 40000r/min respectively. The time of stirring by the homogenizer was 3min.

The LIG-CD inclusion solutions prepared with the above different speed were placed and observed to compare the efficiency of the different stirring speed at room temperature. The LIG-CD inclusion solutions prepared at the speed of 10000 r/min and
15000 r/min began to delaminate obviously after one day. When the rotational speed exceeded 20000 r/min, the LIG-CD liquids prepared were still homogeneous after three days, neither delaminated nor freed. In order to prepare CD inclusion complexes fully and to save energy, 25000 r/min of high speed stirring was finally chosen to prepare the LIG-CD inclusion complexes.

8.3.2.1.3 Selection of Stirring Time

The above mixed solutions of 10g HP-β-CD and 1g LIG were stirred by a homogenizer. The stirring speed of the homogenizer was 25000 r/min. The time of stirring by the homogenizer was 1min, 2min, 3min, 4min, 5min and 10min respectively.

The LIG-CD inclusion solutions prepared with the above different durations were placed and observed to compare the efficiency of the different stirring time at room temperature. The LIG-CD inclusion solutions prepared in 1min and 2min began to delaminate obviously after one day. When the time of stirring exceeded 3min, the prepared LIG-CD solution was still homogeneous after three days, neither delaminated nor free. In order to prepare CD inclusion complexes fully, 4min was finally chosen as the stirring time to prepare the LIG-CD inclusion complexes.

8.3.2.2 Inclusion Ratio Testing of LIG CD Inclusion Solution with UV Method
LIG which un-included by CD cannot dissolve in water because of the low solubility of LIG in aqueous solutions, but LIG which included by CD, thus LIG-CD inclusion complex, can dissolve in water, and the total amount of LIG-CD inclusion complex content quantity is invariable. The prepared LIG-CD was diluted by water, followed by the determination of the solutions’ UV absorbance under the maximum absorption wavelength while the solution of CD was taken as the blank control. The absorbance of the solutions increased with the increase of the added LIG. But when LIG which included by CD was saturated, the absorbance reading graduated into invariableness. Make a graph with the amount of the added LIG as the x-axis and the corresponding absorbency as the y-axis, the concentration of the LIG corresponding to the turning point of the curve would be the maximum inclusion rate of CD to LIG.

UV/VIS Spectrometer Lambda35 (PerkinElmer Instruments, USA) was used to determine the UV absorbance of LIG in the CD inclusion solution. The prepared LIG-CD was diluted by water, and the solution of CD was taken as the blank control. The UV spectrum of LIG in the CD inclusion complex was determined. The result is in Fig 8-6. The maximum absorbance wavelengths were 210nm, 280nm and 330nm respectively. 210nm was the extremity wavelength with much systemic error. So 280nm was chosen as the detected wavelength in this experiment.

8.3.2.2.1 UV Assay of LIG
8.3.2.2 Inclusion Ratio Testing

10g of HP-β-CD was accurately weighed and dissolved in 10ml deionized water at 50°C in a conical flask. The saturated HP-β-CD solution was then allowed to cool down to room temperature. 0.125, 0.25, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0g of LIG were taken and dissolved in 1ml ethanol respectively, and added into the above HP-β-CD solution. Each mixed solution was stirred by a homogenizer. The stirring speed of the homogenizer was 25000r/min. The time of stirring by the homogenizer was 4min. Each prepared solution was diluted by water, and then the UV absorbance of LIG in the CD inclusion solution was determined at 280nm, using the blank HP-β-CD solution as the control. The results were recorded. In order to have a more precise estimation of the inclusion ratio between LIG and HP-β-CD, a graph of the absorbance of LIG versus the amount of LIG used to form inclusion complexes was plotted. From the graph, the saturated point of LIG included by HP-β-CD was found out.

8.3.2.3 Drying Process of Inclusion Solution

Inclusion complexes in solid form can facilitate the application and storage of LIG.
Freeze drying is usually used to dry materials with high heat sensitive. As LIG is heat sensitive, freeze drying was used to dry the LIG inclusion solution.

A freeze drier (Heto Dry Winner Ltd. USA) was used for freeze drying the LIG inclusion solution. Samples of LIG CD inclusion solution were put into a -20℃ freezer for 2h and then into a -80℃ freezer for 30min. When the pressure of the freeze drier became stable, the frozen samples were put into the drying chamber of the drier for freeze drying. The LIG CD inclusion samples were taken out from the drier when they were totally dried, and the drying time was recorded. Then the dried LIG CD inclusion complexes were placed in a desiccator for further use.

8.3.2.4 Validation of LIG CD Inclusion Complexes

Blank solid CD and the freeze dried LIG CD inclusion complex were taken and their crystal structures were observed in a microscope. The crystal structure would change if the LIG was successfully included by CD.

8.3.2.5 LIG Assay of Inclusion Complexes

Solutions Preparation

1.5mg LIG reference was weighted accurately and placed in a 10ml volumetric flask, dissolved and diluted with methanol. And the solution was used as the standard
solution. For the freeze dried LIG CD inclusion complex mentioned before, accurately weighed 0.5g of each inclusion complex in a 100ml volumetric flask, dissolved and diluted it with methanol, and mixed it well and used it as the sample solution.

Assay

HPLC method was used as an assay of the LIG content in the LIG CD inclusion complexes. Please refer to 2.2.3.1.

8.3.2.6 Stability Testing

8.3.2.6.1 High Temperature Testing

The various prepared LIG CD inclusion complex samples were taken into small beakers, which were not specially processed to avoid any influence from oxygen and light. The beakers were placed in ovens under 60°C, and taken out at Day 1, 2.5, 5, 7.5 and 10. After each time period, the contents of LIG were determined using the above HPLC method, and the content variations would be investigated.

8.3.2.6.2 Strong Light Testing

The various prepared LIG CD inclusion complex samples were taken into small beakers, which were not specially processed to avoid any influence from oxygen and
light. The beakers were placed under a 60W daylight lamp at 25℃ respectively, and taken out at Day 1, 2.5, 5, 7.5 and 10. After each time period, the contents of LIG were determined using the above HPLC method, and the content variations would be investigated.

8.3.2.6.3 High Humidity Testing

The various prepared LIG CD inclusion complex samples were taken into small beakers, which were not specially processed to avoid any influence from oxygen. Each beaker was placed in a closed flask with constant humidity 90±5% RH for ten days at 25℃, and wrapped with tinfoil paper to avoid the influence of light, and taken out at Day 1, 2.5, 5, 7.5 and 10. According to the requirements of relative humidity, saturated solutions of KNO₃(92.5 % RH at 25℃) was used. The weight of the substance before and after testing was recorded to evaluate the hygroscopic properties of the substance. After each time period, the contents of LIG were determined using the above HPLC method, and the content variations would be investigated.

8.3.2.7 Solubility Testing

5g of each various prepared LIG CD inclusion complex sample were weighted, and 5ml of deionized water was added into each sample and they were gently shaken for 10s. The degree of dissolution was observed. The time of dissolution was recorded if
the sample was completely dissolved. 0.5 ml of deionized water was added in each sample at an interval of 30 min until the sample was dissolved. The volume of water to completely dissolve the compound in the solubility test must be recorded precisely and the water should be kept at room temperature as the temperature of water can affect the solubility of the compound.

8.4 RESULTS

8.4.1 Preparation Process Optimization of LIG CD Inclusion Solution

In this experiment, the preparation method, the stirring speed and time were chosen and optimized by using single factor test respectively. Finally, high speed stirring was chosen as the method to prepare the LIG-CD inclusion complexes. In order to prepare the CD inclusion complexes fully and to save energy, the speed of 25000 r/min of high speed stirring was finally chosen to prepare the LIG-CD inclusion complexes. And 4min was used for the stirring time to prepare the LIG-CD inclusion complexes.

8.4.2 Inclusion Ratio Testing of LIG CD Inclusion Solution with UV (Table 8-1, Fig 8-7)

According to the UV method, 0.125, 0.25, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0g of LIG were dissolved in 1ml ethanol respectively. After that, these LIG solutions were added into
the HP-β-CD solution which 10g of HP-β-CD was dissolved into 10ml water. Each mixed solution was stirred by a homogenizer at 25000r/min for 4min. Then each prepared solution was diluted by water, and the UV absorbance of LIG in each CD inclusion solution was determined three times each at 280nm. The mean absorbance was recorded. The results were recorded in Table 8-1. The amount of LIG which was included by HP-β-CD was examined with the UV spectrometer. The higher the sample absorbance is, the more LIG included by HP-β-CD is.

Based on the results in Table 8-1, a graph was plotted with LIG amount (X) as the x-axis and UV absorbance (A) as the y-axis, and Fig 8-7 was obtained. The graph showed that the absorbance of LIG increased linearly with the increasing LIG when the added amount of LIG was lower than 1.5g (0.0079mol) with 10g (0.0067mol) HP-β-CD. It indicated that the linear relationship of LIG amount with a range 0-1.5g included by 10g HP-β-CD was reliable. The regression equation with the LIG amount (X) as the x-axis and the UV absorbance (A) as the y-axis was obtained as: A=0.4818X (r=0.9995). And the saturated point of the inclusion process was between 1.5g and 2g LIG with 10g HP-β-CD. After the saturated point of LIG included by HP-β-CD, the inclusion ratio could not be raised by increasing the LIG amount added, and so the curve was flat as no more LIG could be included. Even using more LIG to form inclusion complexes with a fixed amount of HP-β-CD could not increase the absorbance significantly. Based on these results, it was concluded that the inclusion ratio of LIG with HP-β-CD should be about 1:1.
8.4.3 Drying Process of Inclusion Complex

According to the method of the drying process, the samples of LIG inclusion solution were frozen firstly, and then put into a freeze drier to dry. The samples were taken out from the drier when they were totally dried. The drying time was 52h.

8.4.4 Validation of LIG CD Inclusion Complexes (Fig 8-8, 8-9)

The blank solid CD and the freeze dried LIG CD inclusion complex were taken and their crystal structure was observed under a microscope. The results were in Fig 8-8 and Fig 8-9. It showed that LIG CD inclusion complex was the polygonal crystal including oil drip while the blank CD crystal was the round ball structure. The crystal structure was changed when the LIG was included successfully by the CD.

8.4.5 Stability Testing

8.4.5.1 High Temperature Testing (Table 8-2, Fig 8-10)

According to the method of high temperature testing, the prepared LIG CD inclusion complex samples with 0.25, 0.5 and 1g of LIG included by 10g HP- β -CD respectively and control sample(the dried mixture of 0.5g LIG in 10g starch) were
placed in ovens at under 40℃ and 60℃ respectively, and taken out at Day 1, 2.5, 5, 7.5 and 10. After each time period, the contents of LIG were determined using the above HPLC method for three times. The results are shown in Table 8-2 and Fig 8-10.

The results indicated that LIG content of the three different proportional of LIG-CDs was about 68~88% at 60℃ for 10 days, while the LIG content in starch dropped to around 36% under the same condition. The stability of the three different proportional of LIG-CDs was increased at 60℃ obviously when compared to the LIG in starch. So the stability of LIG included by CD was enhanced greatly under the environment of high temperature when the influence of oxygen and light was not absent, while the LIG substance which un-included was degraded largely and fast. With an increased amount of LIG included by HP-β-CD, the content of LIG was decreased gradually. It showed that the LIG was more stable when it was much more strongly included by HP-β-CD. The results indicated that the stability of LIG could be improved by inclusion technology.

8.4.5.2 Strong Light Testing (Table 8-3, Fig 8-11)

According to the method of strong light testing, the prepared LIG CD inclusion complex samples with 0.25, 0.5 and 1g of LIG included by 10g HP-β-CD respectively and control sample (the dried mixture of 0.5g LIG and 10g starch) were placed under a 60W daylight lamp at 25℃ respectively, and taken out at Day 1, 2.5, 5,
7.5 and 10 The contents of LIG in each time periods were determined using the above HPLC method for three times. The results are shown in Table 8-3 and Fig 8-11.

The results indicated that LIG content of the three different proportional LIG-CDs was about 62-80% under light of a 60W daylight lamp at 25℃ for 10 days, while the LIG content in starch dropped to around 45% in the same condition. The stability of the three different proportional LIG-CDs, compared to the LIG in starch, was increased obviously under light of a 60W daylight lamp. So the stability of LIG included by CD was enhanced greatly under the environment of strong light when the influence of oxygen and temperature was not absent, while the LIG which un-included was degraded largely and fast. The results indicated that the stability of LIG could be improved by inclusion technology.

8.4.5.3 High Humidity Testing (Table 8-4, Fig 8-12)

According to the method of high humidity testing, the prepared LIG CD inclusion complex samples with 0.25, 0.5 and 1g of LIG included by 10g HP-β-CD respectively, control sample (the dried mixture of 0.5g LIG and 10g starch) and the blank HP-β-CD were placed in a closed facility with constant humidity about 90±5% RH for ten days at 25℃ respectively, and taken out at Day 1, 2.5, 5, 7.5 and 10. The contents of LIG in different time were determined using the above HPLC method for three times. The results are shown in Table 8-4 and Fig 8-12.
The results indicated that LIG content of the three different proportional LIG-CDs was about 92-97% in humidity about 90±5% RH for ten days at 25℃, while the LIG content in starch dropped to around 65% in the same condition. The stability of the three different proportional LIG-CDs, compared to the LIG in starch, was increased obviously in high humidity. So the stability of LIG included by CD was enhanced greatly under the environment of high humidity when the influence of oxygen and temperature was not absent, while the LIG which un-included was degraded largely and fast. The results indicated that the stability of LIG could be improved by inclusion technology.

The results showed the weight of these samples was increased by about 10% in humidity about 90±5% RH. The increased weight of these samples was similar to the increased weight of the blank HP-β-CD in the same humidity condition. It showed that the HP-β-CD could be affected in high humidity, and LIG could not be affected in high humidity. The humidity influence of HP-β-CD would be resolved by preparation technology.

8.4.6 Solubility Testing (Table 8-5)

According to the method of solubility testing, 5g of the prepared LIG CD inclusion complex samples with 0.25, 0.5 and 1g of LIG included by 10g HP-β-CD
respectively and the blank HP-β-CD was taken in each beaker respectively, and 5ml of deionized water was added into each beaker and shaken gently. Testing was repeated for 3 times. The time and volume of deionized water needed to completely dissolve the samples were recorded and presented in the Table 8-5.

The table 8-5 showed that each LIG CD inclusion complex sample was dissolved by water easily, and the solubility of inclusion complexes of LIG with HP-β-CD was similar to the blank HP-β-CD. The result of the solubility test showed that the inclusion complexes of LIG with HP-β-CD increased the aqueous solubility of LIG and provided a satisfying solubility.

8.5 DISCUSSION

The binding of LIG to HP-β-CD is dependent on the strength of the van der Waals’ force between them. If the van der Waals’ force is weak, then the inclusion complexes will not be stable and thus lead to a low yield of inclusion complexes (Shehatta, 2002). We are unable to control the strength of the van der Waals force between the assigned molecules. However, the performance of the inclusion process can be affected by factors other than the strength of the van der Waals’ force, such as the process method of preparing the inclusion complexes and the proportion of guest molecule to host molecule. Several methods were used to prepare the inclusion complexes. High speed stirring is the most suitable preparation method for LIG-HP-β-CD mixture solution in
laboratory condition as the least re-suspend droplet of LIG occurred in the samples prepared by this method. And we had tried different proportions of LIG to HP-β-CD to test what proportion could give the highest inclusion ratio, and the best one was turned out to be 1:1 as molecular ratio.

Improving the chemical stability and aqueous solubility of LIG is a major aim of preparing LIG-CD inclusion complexes in this experiment. As LIG has poor solubility in aqueous solutions, this problem could be settled through inclusion experiment of CD. Certainly, we hope that LIG could be included by CD as much as possible. According to the properties of CD inclusion complexes, the extent of improvement in stability to drugs with poor stability is directly related to the inclusion efficiency, which is related to the inclusion ratio between the host molecule and the guest molecule. Therefore, the inclusion ratio greatly affects the quality of LIG–CD inclusion complexes.

The stability of the three different proportional LIG-CDs, compared with the LIG in starch, was increased obviously. The stability of LIG included by HP-β-CD was greatly enhanced under the environment of high temperature, strong light and high humidity, while the LIG un-included by HP-β-CD was degraded largely and quickly. This suggested that it be completely viable to improve the stability of LIG inclusion by HP-β-CD. Besides, we didn’t remove the oxygen from the samples when the accelerating experiment was carried out, which meant the effect of air existed in the
accelerating experiment. But, actually, the influence of oxygen to the stability of LIG could be eliminated if proper oxygen-excluding application was undertaken, thus LIG’s stability in LIG CD inclusion complexes would be improved more greatly.

LIG itself is aqueous-insoluble, but when it is included by HP-β-CD, it becomes aqueous-soluble. Thus the UV absorbance of LIG can't be displayed because it couldn’t be dissolved in water, while the situation will be quite opposite if it is with inclusion. As a control, when LIG was in starch, the UV absorbance was nearly 0. And the UV absorbance of the LIG CD inclusion complexes increases with the increase of the amount of included LIG until the inclusion achieve saturation, at then the UV absorbance will be the maximal value. In this experiment, however, it might not reach this ideal maximum value. The theoretical maximal amount of LIG at saturation can be calculated by the linear regression equation of the absorption curve, as the absorption value of LIG at saturation is known. This is a new way to detect and calculate the maximal amount of drug for the inclusion of HP-β-CD.

Through this experiment, it was proved that the inclusion LIG by CD was completely feasible. LIG is an active molecule that is easily denatured by high temperature or oxidized. This situation can be improved by forming inclusion complexes with HP-β-CD when the active site that is easily attack by foreign compounds is covered by HP-β-CD. The LIG-CD inclusion complexes prepared according to the preparation process not only increased the stability of LIG, but also made it dissolve in water fully,
showing that the inclusion preparation of CD was suitable for LIG. Meanwhile, the liquid LIG is transformed into solid so that it could be used as the raw drug materials for oral tablets, powder for injection and so on, which will surely widen the application of LIG preparations remarkably.
**Figure 8-1.** Chemical structure and torus shape of β-CD molecule

**Figure 8-2.** 3-D structure of β-CD
Figure 8-3. Structure of HP-β-CD (R is HP being replaced in β-CD derivative)

Figure 8-4. Formation of 1:1 inclusion complex

Figure 8-5. Formation of 1:2 inclusion complex
Figure 8-6. The UV spectrum of LIG CD inclusion complex. The solution of HP-β-CD was taken as the blank control. The maximum absorbance wavelength was 210nm, 280nm, 330nm respectively. 210nm is the extremity wavelength with much systemic error. So 280nm was chosen as the detected wavelength in this experiment.

Table 8-1. UV Absorbance of LIG CD inclusion solution samples at 280nm

<table>
<thead>
<tr>
<th>(g)</th>
<th>0.125</th>
<th>0.25</th>
<th>0.5</th>
<th>1</th>
<th>1.5</th>
<th>2</th>
<th>2.5</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absorbance (A)</td>
<td>0.052±0.005</td>
<td>0.116±0.008</td>
<td>0.248±0.003</td>
<td>0.492±0.002</td>
<td>0.715±0.001</td>
<td>0.795±0.002</td>
<td>0.825±0.003</td>
<td>0.827±0.001</td>
</tr>
</tbody>
</table>
**Figure 8-7.** Graph of absorbance vs. LIG added for inclusion process. A graph was plotted with LIG amount (X) as the x-axis and UV absorbance (A) as the y-axis. It indicated that the linear relationship of within 0-1.5g LIG included by 10g HP-β-CD was reliable. The regression equation of LIG amount (X) as the x-axis and UV absorbance (A) as the y-axis was obtained as: \( A = 0.4818X \) \((r=0.9995)\). And the saturated point of inclusion process was between 1.5g and 2g LIG with 10g HP-β-CD. After the saturated point of LIG included, the inclusion ratio could not be raised by increasing the LIG amount added so the curve was flat as no more LIG could be included. Even using more LIG to form inclusion complexes with a fix amount of HP-β-CD cannot increase the absorbance significantly. Based on these results, it was concluded that the inclusion ratio of LIG with HP-β-CD should be about 1:1.
Figure 8-8. The photo of the blank HP-β-CD. The crystal of blank HP-β-CD is the ball model.

Figure 8-9. The photo of the LIG CD inclusion. The LIG CD inclusion complex was the polygonal crystal including oil drip.
**Table 8-2.** The results of high temperature testing.

(Mean ± S.D. of three measurements)

<table>
<thead>
<tr>
<th>LIG:CD</th>
<th>1d</th>
<th>2.5d</th>
<th>5d</th>
<th>7.5d</th>
<th>10d</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5:10</td>
<td>86.8±1.9</td>
<td>81.7±2.2</td>
<td>76.1±1.8</td>
<td>69.9±1.2</td>
<td>68.3±1.7</td>
</tr>
<tr>
<td>0.5:10</td>
<td>96.1±1.2</td>
<td>91.9±1.4</td>
<td>86.3±2.1</td>
<td>80.1±2.8</td>
<td>76.7±1.3</td>
</tr>
<tr>
<td>0.25:10</td>
<td>97.6±1.8</td>
<td>95.6±2.5</td>
<td>92.9±1.3</td>
<td>91.8±2.0</td>
<td>88.3±2.1</td>
</tr>
<tr>
<td>Control</td>
<td>75.3±2.1</td>
<td>61.6±1.7</td>
<td>54.4±0.9</td>
<td>44.9±1.5</td>
<td>36.6±2.5</td>
</tr>
</tbody>
</table>

**Figure 8-10.** The results of high temperature testing. The results indicated LIG content of the three different proportional LIG-CDs was about 68~88% at 60 °C for 10 days, while the LIG content in starch dropped to around 36% at the same condition. The stability of the three different proportional LIG-CDs, compared to the LIG in starch, was increased obviously at 60°C. So the stability of LIG included by CD was enhanced greatly under the environment of high temperature when the influence of oxygen and light was not absent, while the LIG substance which un-included was degraded largely and fast. The results indicated that the stability of LIG could be improved by inclusion technology.
Table 8-3. The results of strong light testing

(Mean ± S.D. of three measurements)

<table>
<thead>
<tr>
<th>LIG:CD</th>
<th>1d</th>
<th>2.5d</th>
<th>5d</th>
<th>7.5d</th>
<th>10d</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5:10</td>
<td>82.1±2.1</td>
<td>72.9±1.9</td>
<td>68.4±2.1</td>
<td>64.3±3.2</td>
<td>62.2±2.4</td>
</tr>
<tr>
<td>0.5:10</td>
<td>93.2±2.4</td>
<td>84.1±3.1</td>
<td>80.4±2.5</td>
<td>78.0±1.8</td>
<td>74.1±3.3</td>
</tr>
<tr>
<td>0.25:10</td>
<td>95.4±1.8</td>
<td>91.4±2.7</td>
<td>87.9±0.9</td>
<td>83.1±2.7</td>
<td>79.7±3.1</td>
</tr>
<tr>
<td>Control</td>
<td>78.3±1.2</td>
<td>69.6±1.5</td>
<td>60.1±1.8</td>
<td>49.3±1.6</td>
<td>38.4±1.4</td>
</tr>
</tbody>
</table>

Figure 8-11. The results of strong light testing. The results indicated that the LIG content of the three different proportional LIG-CDs was about 62-80% under light of a 60W daylight lamp at 25°C for 10 days, while the LIG content in starch dropped to around 45% in the same condition. The stability of the three different proportional LIG-CDs, compared to the LIG in starch, was increased obviously under light of a 60W daylight lamp. So the stability of LIG included by CD was enhanced greatly under the environment of strong light when the influence of oxygen and temperature was not absent, while the LIG substance which un-included was degraded largely and fast. The results indicated that the stability of LIG could be improved by inclusion technology.
### Table 8-4. The results of high humidity testing

(Mean ± S.D. of three measurements)

<table>
<thead>
<tr>
<th>LIG:CD</th>
<th>1d</th>
<th>2.5d</th>
<th>5d</th>
<th>7.5d</th>
<th>10d</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5:10</td>
<td>99.8±0.9</td>
<td>97.6±1.1</td>
<td>95.7±1.4</td>
<td>93.8±2.4</td>
<td>92.2±2.1</td>
</tr>
<tr>
<td>0.5:10</td>
<td>99.8±0.6</td>
<td>98.6±1.3</td>
<td>96.4±2.2</td>
<td>95.2±2.6</td>
<td>94.0±1.9</td>
</tr>
<tr>
<td>0.25:10</td>
<td>99.9±0.4</td>
<td>98.9±2.1</td>
<td>98.1±1.8</td>
<td>97.8±1.4</td>
<td>97.1±2.1</td>
</tr>
<tr>
<td>Control</td>
<td>95.1±1.2</td>
<td>89.3±2.3</td>
<td>81.8±1.5</td>
<td>75.4±2.3</td>
<td>65.5±2.8</td>
</tr>
</tbody>
</table>

![Figure 8-12](image.png)

**Figure 8-12.** The results of high humidity testing. The results showed that the weight of these samples was increased about 10% in humidity about 90±5% RH. The increased weight of these samples is similar to the increased weight of the blank HP-β-CD in different humidity condition. It showed the HP-β-CD could be affected in high humidity, and LIG could not be affected in high humidity. The humidity influence of HP-β-CD will be resolved by preparation technology.
Table 8-5. The results of solubility Testing

<table>
<thead>
<tr>
<th>Sample (5g)</th>
<th>Blank CD</th>
<th>0.25 LIG-CD</th>
<th>0.5 LIG-CD</th>
<th>1.5 LIG-CD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (min)</td>
<td>20±2</td>
<td>20±2</td>
<td>22±3</td>
<td>23±3</td>
</tr>
<tr>
<td>Water volume</td>
<td>5ml</td>
<td>5ml</td>
<td>5ml</td>
<td>5ml</td>
</tr>
</tbody>
</table>

It showed that each LIG CD inclusion complex sample was easily dissolved by water, and the solubility of inclusion complexes of LIG with HP-β-CD was similar to the blank HP-β-CD. The result of solubility test showed the inclusion complexes of LIG with HP-β-CD increased the aqueous solubility of LIG and provided a satisfying solubility.
CHAPTER 9

MICRO-EMULSION PREPARATION OF LIGUSTILIDE

9.1 ABSTRACT

LIG micro-emulsion (ME) preparation was studied by adopting soybean phosphatide as an emulsifier, ethanol as a co-emulsifier, olive oil as an oil phase, and ascertaining the optimal forming region of ME by plotting the pseudo-ternary phase diagram. The optimum proportion and method of LIG ME preparation was obtained after a series of the preparation process experiments. It was found that the stability of LIG would increase greatly upon the optimum inclusion ratio and well prepared process. The method of LIG ME could establish a solid foundation for LIG to develop new pharmaceutical preparations.

9.2 INTRODUCTION

In recent years, ME has become one of the hot subject in the pharmaceutical industry. It could improve the bioavailability of drug absorbed in vivo (Cheng et al., 1995). The stability of ME can extend the shelf life of drug and accelerate its diffusion as well as absorption. Therefore, the drug delivery systems of ME have considerable potential in
the study of pharmaceutics. The different types of ME can transform into each other under some objective condition because of its changeable internal structure. ME system has been proposed to enhance the bioavailability of drugs and to develop a time-released, fast-released and targeting administration of drugs.

ME, a thermodynamic stable system with single phase transparence, well liquidity and isotropy, is composed of surfactant, co-surfactant, oil and water. Studying the phase behaviors and structure properties of ME was very important in the pharmaceutical industry (Palani et al., 1995). The uniform single phase ME has been studied more widely and deeply, as its dispersed phase is composed of uniform nanometer droplets. It can be classified into two kinds according to the compositions of the continuous phase and disperse phase: the normal phase ME is oil-in-water (O/W), while the reversed one is water-in-oil (W/O). The hydrophile lipophile balance (HLB) value is a primary index to the formulation design of ME. Generally speaking, it is easy to form O/W ME when the HLB value of the system is between 3 and 6, while it is favorable to form O/W ME when the HLB value is between 8 and 18. The HLB value of the system is decided by the kind and quantity of the surfactant and co-surfactant.

The normal size of ME is between 10nm and 100nm (Hague et al., 1988). It is a transparent liquid uniform in shape, and most of the ME drops are globular with a few being cylindrical. As a thermodynamic stable system, ME wouldn’t delaminate when
heated or centrifuged. It will be destroyed at the critical point of phase when the temperature of ME is changed, but the destroyed ME will become normal state if the temperature returns to origin. It was usually necessary to add some co-emulsifier, and better to gently mix it up with ME when preparing. ME itself is a micelle solution, but it quite differs from others in both composition and structure though they might have similar appearance. Oil-in-water ME couldn’t be illimitably diluted by water, and there was no obvious quantitative relationship between the solubility of oil and the emulsifier.

What makes ME a fundamental in the pharmaceutical field, maintains the high stability and eases the preparation, is the ability to considerably increase the bioavailability of lipophilic drug and to speed up the drug-uptake by liver for lipophilic drug to get into hydrophilic carriers through the loading process. The great variety applications of ME have offered numerous subjects to the study of ME properties, such as the mechanism of drug release. One of the most important properties of the ME drug delivery system is the release profile, which describes how drugs diffuse out of the ME carrier after administration, not only in close correlation to drug transport and metabolism in vivo, but also deeply reflecting the stability of ME. The mechanism of drug release of ME is very complicated. It is very difficult to propose a complete quantitative theoretical formula about drug release from ME. A number of experiment methods for the determination of release profiles from disperse systems have been used till now. All have their advantages and disadvantages.
Generally, drug release from ME acts as inverse ratio to the stability of ME. There are several types of drug release by adjusting inside osmotic pressure and outside water phase (Gan et al., 1994). And the release rate of drug from ME is determined by the type and concentration of the surfactant in ME.

Though ME is able to be formed spontaneously without any external action, experiments show that, increasing the stirring speed and prolonging the stirring time properly could help to reduce the amount of emulsifier and co-emulsifier to obtain the same good effect. Thus, we should pay more attention to the stirring speed and time when preparing ME. The preparation should be observed when adding the materials with the sequence of emulsifier, co–emulsifier, oil phase and water. And the water should be added slowly. Otherwise irreversible turbidity would be easily caused. More emulsifier and co-emulsifier would be needed if the oil phase and water phase were mixed before adding the emulsifier and co-emulsifier.

Considering ME has so many strong points to enhance the stability and improve the absorption of drug, ME is a suitable preparation to LIG according to the analysis and research to physical and chemical properties of LIG. In this experiment, we have studied the preparation process of LIG ME with LIG as the drug and ME as the carrier. In addition, the pharmaceutical properties of ME have been studied through examining and observing its physical and chemical properties. We hope to obtain a stable LIG ME preparation, which could be used for oral administration and
intravenous injection solution of LIG.

9.3 MATERIALS AND METHODS

9.3.1 Materials

9.3.1.1 Materials

Soybean oil, olive oil, colza oil and Pluronic F68 were purchased from BASF Chemical Co. Germany. Soybean Phosphatide, PEG 400, Tween-20 and Tween-80 were from Sigma Chemical Co. USA. Anhydrous Ethanol was from Bio-Rad Technology Ltd., USA. HPLC grade methanol was from Honeywell International Inc., Muskegon, USA. Isopropyl alcohol was from Tedia Company, Fairfield, USA. Deionized water was generated from a Milli-Q water system (Millipore, Bedford, MA, USA).

9.3.1.2 LIG Preparation

LIG was extracted, separated and purified from Radix *Angelica sinensis* in our laboratory, with batch number: 050118. Purified LIG was identified by electron impact ionization (EI) MS, $^1$H NMR and $^{13}$C NMR spectrometric techniques. The purity was found to be > 98% based on the percentage of total peak area by GC
9.3.2 METHODS

9.3.2.1 Optimization of Preparation Process for LIG ME

9.3.2.1.1 Selection of Emulsifier

In the preparation of ME, the properties, toxicity as well as the cost of emulsifier should be taken into consideration. In this experiment, Tween-20, Tween-80, PEG-400, Pluronic F-68 and soybean phosphatide were studied as candidate emulsifier. The results indicated that when Tween-20, Tween-80, PEG-400 and Pluronic F-68 were mixed with the co-emulsifier short chain alcohols (ethanol, isopropanol, glycerin or n-butanol) in different proportions, they segmented and turned into a milky fluid that was unable to form ME. But soybean phosphatide could form a transparent and clear ME with ethanol, isopropanol, glycerin or N-butanol. Soybean phosphatide is a natural amphoteric ion surfactant with low toxicity, and its usual volume is 10-34g/L. Refined soybean phosphatide that contains such polar radicals as phosphate radical, choline and hydrocarbon chains are lipophilic and slightly oleophilic, so it can be used for the preparation of intravenous injection fluids. Therefore, soybean phosphatide was selected as the emulsifier for LIG ME.
9.3.2.1.2 Selection of Co-emulsifier

Though soybean phosphatide can form a transparent and clear ME with ethanol, isopropanol, glycerin or n-butanol, however, isopropanol, glycerin and N-butanol are proven to be high toxic, ethanol was therefore selected as the co-emulsifier for its low toxicity, good solubility and capacity to form stable O/W ME.

9.3.2.1.3 Selection of Oil Phase Material

Soybean oil, colza oil and olive oil were respectively added into LIG and surfactant, mixed up each match till it became a transparent yellow fluid. Then deionized water was slowly added into the mixture, mixed thoroughly and observed immediately.

**Soybean Oil**

The appearance of the soybean oil mixture first turned into transparent yellow, and then into cloudy yellow, latter into milky cloudy white when the deionized water was continually added into the mixture. The diameter of the mixture was measured with a microscope throughout the process. The average diameter was about ten micrometers which failed to meet the criteria of nanometer grade. No double refraction was observed under a polarized microscope.

**Colza Oil:**
The appearance of the colza oil mixture turned from a transparent yellow into cloudy yellow, transparent yellow, cloudy yellow and finally into cloudy milky when the deionized water was continually added into the mixture. The diameter of the mixture was measured with a microscope throughout the process. The average diameter was about several micrometers which also failed to meet the criteria for nanometer grade. No double refraction was observed under a polarized microscope.

**Olive Oil**

The appearance of the olive oil mixture turned from a transparent yellow into cloudy yellow, transparent yellow, cloudy yellow, cloudy milky and finally into yellow gel when the deionized water was continually added into the mixture. The diameter of the mixture was measured with a microscope throughout the process. The average diameter was about several hundred nanometers which met the criteria for nanometer grade. Double refraction was observed under a polarized microscope. Through the comparison, the mixture of olive oil and LIG had the best effect for LIG ME, thus olive oil was selected as the oil phase for LIG.

**9.3.2.1.4 Selection of Ratio of Emulsifier and Co-emulsifier**

Soybean phosphatide (emulsifier) and ethanol (co-emulsifier) were respectively taken with the ratio of 1:1, 1:1.5, 1:2. After that, added them into the equal mixture of oil phase and LIG which had the ratio as 1:1, and mixed them well. Then deionized water
was added by drips during the eddying shaking and the phenomenon that the mixture turned from clear into cloudy or from cloudy into clear was observed. With a microscope to observe the size of latex grains and the phenomenon of each sample, we found that the samples mixed with the ratio of 1:1.5 and 1:2 would delaminate except the one with ratio of 1:1 after each sample was laid aside for a long time. Therefore, we chose the ratio 1:1 to mix the emulsifier and co-emulsifier.

### 9.3.2.1.5 Phase Diagram Preparation

ME is a mixture composed of multi-components, with at least three but usually four to five components. Single, double or triple phase can be obtained by changing the parameters of the system. Phase diagram is the basic tool to study ME. The determination of the phase border is very important in the study of ME. We should distinguish the ME phase, liquid crystal phase, common emulsion phase and gel phase when the phase diagram was prepared. The gel phase had high viscosity and poor liquidity. And the common emulsion phase was an ivory-white opaque liquid. As for the ME phase and liquid crystal phase, they were both transparent, clear liquids with good liquidity, which needed polarizing optical microscopy (POM) to distinguish--the one with double-fold phenomenon was the liquid crystal phase, while the other one without was the ME phase.

Pseudo-ternary phase diagram was plotted by taking an emulsifier (soybean phosphatide) and a co-emulsifier (anhydrous ethanol) as the emulsifier phase, olive oil
and LIG as the oil phase, and deionized water as the aqueous phase. At 40°C, emulsifier phase the oil phase and aqueous phase in different ratios were taken into a beaker and mixed thoroughly, then the phenomenon change was observed with the color transferred from clear to thick and then back to clear. The percentage of all components at critical points was recorded. Then pseudo-ternary phase diagram was drawn with the percentage of all components at critical points. The optimum LIG ME formulation would be obtained at the O/W ME zone in the pseudo-ternary phase diagram.

9.3.2.2 Quality Tests of ME

9.3.2.2.1 Primary Test of ME

The transparent or semitransparent water-oil-surfactant dispersant system with fine fluidity can be considered as ME if phase separation won’t take place under the condition of centrifugation at 10000r/min for 5 min. So the primary test for ME was the centrifugation at 10000r/min for 5 min. After centrifugation, the ME was observed to check the presence of separation. If the ME separated into layers, it indicates that the preparation of ME was not successful.

9.3.2.2.2 Determination of Particle Size and Distribution
The size of the ME prepared should be controlled to ensure it was small enough for usage. The range of the ME required was between 0.1\(\mu\)m and 2.0\(\mu\)m diameter. The determination of the ME diameter was done by a laser dynamic light-scattering particle sizer. By the use of the laser dynamic light-scattering particle sizer (Nano Sz 900), the size distribution and the average diameters of ME could be determined.

### 9.3.2.3 LIG Assay of LIG ME

**Solutions Preparation**

Accurately weighed 1.5mg LIG reference in a 10ml volumetric flask, dissolved and diluted with methanol, mixed well and used as the standard solution. Accurately weighed 0.5g of each LIG ME in a 100ml volumetric flask, dissolved and diluted with methanol, mixed well and used as the sample solution.

**Assay**

HPLC method was used as assay of the LIG content in LIG ME. Please refer to the HPLC method in 2.2.3.1.

### 9.3.2.4 Stability Test of LIG ME Preparation

#### 9.3.2.4.1 Preparation of Test Solution

According to the optimum formulation of LIG ME from the pseudo-ternary phase
9.3.2.4.2 Accelerated Stability Testing

9.3.2.4.2.1 High Temperature Testing

The various prepared LIG ME samples were taken into small centrifuge tubes, which were sealed to prevent the samples from evaporation for heating. The tubes were placed in ovens under 60°C for 10 days, and taken out at Day 1, 2.5, 5, 7.5 and 10 respectively. The physical stability of LIG ME at different time was tested under the condition of centrifugation at 10000r/min for 5 min. The content of LIG in LIG ME at different time was determined using the above HPLC method, and the LIG content variation would be investigated.

9.3.2.4.2.2 Strong Light Testing

The various prepared LIG ME samples were taken into small centrifuge tubes. The processed centrifuge tubes were placed under a 60W daylight lamp at room temperature for 10 days, and taken out at Day 1, 2.5, 5, 7.5 and 10 respectively. The physical stability of LIG ME at different time was tested under the condition of
centrifugation at 10000r/min for 5 min. The content of LIG in LIG ME at different
time was determined using the above HPLC method, and the LIG content variation
would be investigated.

9.4 RESULTS

9.4.1 Optimization of Preparation Process for LIG ME

9.4.1.1. Optimization Condition of LIG ME

According to the single factor experiment of preparation process for LIG ME, the
optimum emulsifier was soybean phosphatide, the optimum co-emulsifier was ethanol,
the optimum ratio of emulsifier and co-emulsifier was 1:1 and the optimum oil phase
material was olive oil.

9.4.1.2 Phase Diagram Preparation (Fig 9-1)

According to the preparation method of phase diagram, the emulsifier phase of
soybean phosphatide and anhydrous ethanol (1:1) and the oil phase of olive oil and
LIG (1:1) by the ratios of 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8 and 1:9 were taken in a
series of beakers, respectively. Changes of the mixture from clear to thick and then
from thick to clear were observed while adding deionized water by drips during the
mixing. Then we could draw plot for pseudo-ternary phase diagram by recording the percentage of all components at critical point. The first critical point of the ME was at the moment when the mixed solution changed from clear to thick. The change from thick to clear was the second critical point. Finally the change from clear into thick was the third critical point. Pseudo-ternary phase diagram of LIG ME is in Fig 9-1. There was only one critical point when the ratios of emulsifier phase to oil phase were 9:1, 8:2, 3:7, 2:8 and 1:9, and, for these 5 ratios, the mixture couldn’t transfer from thick to clear even by adding water illimitably behind the critical point. However, there were three critical points when the ratios of emulsifier phase to oil phase were 7:3, 6:4, 5:5 and 4:6, but the mixture also couldn’t transfer from thick to clear by adding water illimitably behind the each critical point. Moreover, the stability O/W zone of LIG ME could be formed in the ratio range between 7:3 and 4:6. The optimum LIG ME formulation could be obtained at the O/W ME zone in the pseudo-ternary phase diagram.

9.4.2 Quality Tests of ME

9.4.2.1 Primary Test of ME

The primary emulsion obtained went through centrifugation at 10000r/min for 5 minutes. No separation into layers of the ME prepared was observed. This showed that the preparation of emulsion was successful.
9.4.2.2 Determination of Particle Size and Distribution (Fig 9-2, Fig 9-3)

The determination of the ME diameter was done by a laser dynamic light-scattering particle sizer. 20ml of ME sample was pretreated by adding into 800ml water at 25°C. By the use of the laser dynamic light-scattering particle sizer (Nano Sz900), the size distribution and the average diameters of ME can be determined. The result is in Fig 9-2 and Fig 9-3. It indicated the diameter range of the prepared ME was from 0.1µm to 1µm and the average diameter was about 270nm. The diameter of the prepared ME was acceptable.

9.3.2.3 Stability Test of LIG ME Preparation

9.3.2.3.1 High Temperature Testing (Table 9-1 and Fig 9-4)

According to the method of high temperature testing, three LIG ME samples containing 5%, 10% and 20% LIG and the control sample (5% LIG was dissolved in olive oil) were placed in ovens under 60°C and taken out at Day 1, 2.5, 5, 7.5 and 10. The LIG ME samples at different time went through centrifugation at 10000r/min for 5 minutes. There was no separation into layers in each LIG ME sample. This showed that the physical stability of LIG ME was very good in high temperature. The content of LIG in LIG ME at different time was determined using the above HPLC method.
The results indicated the LIG content of the three different proportional LIG ME was about 60~70% at 60 °C for 10 days, while the LIG content in olive oil dropped to around 35% at the same condition. The stability of LIG in the three different ME, compared to the LIG in olive oil, was increased obviously at 60°C. So the stability of LIG in ME was enhanced greatly under the environment of high temperature when the influence of oxygen and light was not absent. The results indicated the stability of LIG could be improved by ME technology.

**9.3.2.3.2 Strong Light Testing (Table 9-2, Fig 9-5)**

According to the method of strong light testing, three LIG ME samples containing 5%, 10% and 20% LIG and the control sample (5% LIG was dissolved in olive oil) were placed under a 60W daylight lamp at 25°C respectively, and taken out at Day 1, 2.5, 5, 7.5 and 10. The LIG ME samples at different time went through centrifugation at 10000r/min for 5 minutes. There was no separation into layers in all LIG ME samples. This showed the physical stability of LIG ME was very good in strong light condition. The content of LIG in LIG ME at different time was determined using the above HPLC method for three times. The results are shown in Table 9-2 and Fig 9-5.

The results indicated that the LIG content of the three different LIG ME was about
74-82% under a 60W daylight lamp at 25°C for 10 days, while the LIG content in olive oil dropped to about 50% at the same condition. The stability of LIG in the three different ME, compared to the LIG in olive oil, was increased obviously under a 60W daylight lamp. So the stability of LIG in ME was enhanced greatly under the environment of strong light when the influence of oxygen and temperature was not absent. The results indicated the stability of LIG could be improved by ME technology.

9.5 DISCUSSION

The first procedure is to determine the optimum conditions of ME preparation, which include the ratio between emulsifiers and co-emulsifiers as well as the ratio between oil phase and aqueous phase. In the pseudo-ternary phase diagram of ME, it shows that the ratio among oil, water and emulsifiers is important to form stable ME. Although the volume of consumed water, oil and emulsifiers are not very important, several trials must be performed to determine the optimum ratio among oil, water, emulsifiers and co-emulsifiers. In this project, three ratios were tried separately to obtain the optimum ratio and volume of the solvents used.

The formulation design of ME relies on the proper selection and ration of components to enable the fitness of the components for pharmaceutical purpose and ME formation in a wide range. One of the difficulties is that the prepared ME should be of low
viscosity, preferred to be stable and rheological in terms of Newtonian Fluid, with a small and evenly-distributed diameter and good inclusive property. In the preparation of ME, single factor tests were adopted to determine the best emulsifier, the best oil phase and the ratio of emulsifier phase as well as the oil phase. A phase diagram for the emulsifier phase, oil phase and water phase was made in this study. Phase diagram is the basic tool for the study of ME. ME is a mixture composed of multi-components, with at least three, usually four or five components. Single, double or triple phase can be obtained by changing the parameters of the system. The determination of the phase border is very important in the study of ME.

There are two basic conditions for the formation of ME. First, the mixture of emulsifier and co-emulsifier should adequately adsorb at the oil-water interface, which means that the HLB of the emulsifier employed must match with the practical system. Second, the interface should be flexible enough. To obtain the above mentioned two conditions, we can add co-emulsifier into ionic surfactant or adjust the temperature of non-ionic surfactant. Since the emulsifier and co-emulsifier in the ME had high concentration, these two excipients selected by present study were biologically compatible materials without any toxicity and side effects.

As far as selection of emulsifier is concerned, we should take the biocompatibility and toxicity of the emulsifier into account apart from its effects. The usage of emulsifier is the most important factor in terms of drug-loading. The more emulsifier is used, the
more drugs will be loaded. Emulsifier also affects the size and distribution of grains. To ensure that there will be no crystal formation in ME, try to use less emulsifier as you can. In order to enhance the stability of ME, we should choose the temperature range of the ME preparations as wide as possible. The presence of drugs affects the range of ME formation, also has effect to the ME zone to some extent, which suggests an interaction between drug molecule and the interface of ME.

Stability evaluation is a basic tool to assess the shelf life of ME as well as its quality. Currently there is no well-established method to assess the stability of ME. Transparent or semitransparent water-oil-surfactant dispersant systems with fine fluidity can be considered as ME if phase separation won’t take place in the condition of centrifugation at 10000 r/min for 5 min. The results of centrifugal experiment indicate the stability of ME while measuring the size and distribution variation of grains.
**Figure 9-1.** Pseudo-ternary phase diagram of LIG ME. There was only one critical point when the ratios of emulsifier phase to oil phase were 9:1, 8:2, 3:7, 2:8 and 1:9, and, for these 5 ratios, the mixture couldn’t transfer from thick to clear even by adding water illimitably behind the critical point. However, there were three critical points when the ratios of emulsifier phase to oil phase were 7:3, 6:4, 5:5 and 4:6, but the mixture also couldn’t transfer from thick to clear by adding water illimitably behind the each critical point. Moreover, the stability O/W zone of LIG ME could be formed in the ratio range between 7:3 and 4:6. The optimum LIG ME formulation could be obtained at the O/W ME zone in the pseudo-ternary phase diagram.
Figure 9-2. The particle size and distribution of LIG ME. The diameter range of the prepared ME was from 0.1µm to 1µm and the average diameter was about 270nm. The diameter of the prepared ME was acceptable.
Figure 9-3. The micrograph of LIG ME

Table 9-1. The results of high temperature test.
(Mean ± S.D. of three measurements)

<table>
<thead>
<tr>
<th>LIG content</th>
<th>1d</th>
<th>2.5d</th>
<th>5d</th>
<th>7.5d</th>
<th>10d</th>
</tr>
</thead>
<tbody>
<tr>
<td>5%</td>
<td>97.7±2.0</td>
<td>91.7±1.3</td>
<td>83.6±1.6</td>
<td>75.7±1.7</td>
<td>70.6±1.4</td>
</tr>
<tr>
<td>10%</td>
<td>96.7±1.8</td>
<td>90.4±2.3</td>
<td>78.7±1.8</td>
<td>68.8±2.1</td>
<td>63.5±0.9</td>
</tr>
<tr>
<td>20%</td>
<td>94.9±1.4</td>
<td>88.5±2.1</td>
<td>75.2±2.3</td>
<td>66.1±1.3</td>
<td>60.4±1.5</td>
</tr>
<tr>
<td>Control (5%)</td>
<td>85.7±1.5</td>
<td>74.3±1.5</td>
<td>59.3±2.1</td>
<td>46.3±2.2</td>
<td>35.9±1.8</td>
</tr>
</tbody>
</table>
Figure 9-4. The results of high temperature tests. The results indicated that the LIG content of the three different proportional LIG ME was about 60~70% at 60 °C for 10 days, while the LIG in olive oil dropped to around 35% at the same condition. The stability of LIG in the three different ME, compared to the LIG in olive oil, was increased obviously at 60°C. So the stability of LIG in ME was enhanced greatly in high temperature when the influence of oxygen and light was not absent. The results indicated the stability of LIG could be improved by ME technology.

Table 9-2. The results of strong light testing

(Mean ± S.D. of three measurements)

<table>
<thead>
<tr>
<th>LIG content</th>
<th>1d</th>
<th>2.5d</th>
<th>5d</th>
<th>7.5d</th>
<th>10d</th>
</tr>
</thead>
<tbody>
<tr>
<td>5%</td>
<td>98.4±2.1</td>
<td>95.5±1.9</td>
<td>91.8±2.7</td>
<td>86.5±1.1</td>
<td>82.2±1.5</td>
</tr>
<tr>
<td>10%</td>
<td>96.7±1.2</td>
<td>91.7±2.1</td>
<td>84.7±1.4</td>
<td>79.7±2.5</td>
<td>75.8±2.1</td>
</tr>
<tr>
<td>20%</td>
<td>95.9±1.8</td>
<td>90.4±2.3</td>
<td>83.7±2.6</td>
<td>78.4±1.2</td>
<td>74.1±0.9</td>
</tr>
<tr>
<td>Control (5%)</td>
<td>92.4±1.3</td>
<td>82.2±2.2</td>
<td>67.4±2.3</td>
<td>57.1±2.1</td>
<td>49.9±2.8</td>
</tr>
</tbody>
</table>
Figure 9-5. The results of strong light testing. The results indicated that the LIG content of the three different LIG ME was about 74-82% under a 60W daylight lamp at 25°C for 10 days, while the LIG content in olive oil dropped to about 50% at the same condition. The stability of LIG in the three different ME, compared to the LIG in olive oil, was increased obviously under a 60W daylight lamp. So the stability of LIG in ME was enhanced greatly under the environment of strong light when the influence of oxygen and temperature was not absent. The results indicated the stability of LIG could be improved by ME technology.
CHAPTER 10

ACUTE TOXICITY TEST OF LIGUSTILIDE

10.1 ABSTRACT

In this study, the acute toxicity of LIG had been studied. Acute toxicity test used index LD$_{50}$ to define the intrinsic acute toxicity of the high pure LIG with NIH mouse. The LD$_{50}$ was calculated by software LD$_{50}$ with the method Bliss which adopted the mortality rate. The result indicated that LIG was safe to use as a drug because the LD$_{50}$ of intraperitoneal injection (i.p.) and intragastrical injection (i.g.) group was 259.04 mg/kg and 649.71 mg/kg respectively. From the results, it could be concluded that the toxicity of LIG is of a low level. It was mainly used for providing information for long-term toxicity studies.

10.2 INTRODUCTION

The objectives of acute toxicity test are to define the intrinsic toxicity of the chemical, identify the target organs, and predict the hazard to non-target species. This can not only provide information for risk evaluation of acute exposure to the testing chemical, but also provide information for the design and selection of future studies on dose level. Most importantly, it also provides information for clinicians about acute
over-exposure or poisoning. Acute toxicity studies are the first defending line before long-term studies (Davis, 2002). The acute toxicity data is essential in the classification, labeling of chemical in regulatory standpoint. In the academic point of view, acute toxicity studies always provide important clues on mechanisms and structure activity relationship for the tested chemicals (Gad and Chengelis, 1998).

The test substance is given in diluted form at different dosages. It is diluted with appropriate solvent such as 3% Tween-80. Clinical signs, morbidity and mortality are observed. Animals that die or become moribund during the study are subjected to necropsies, while for those survive throughout the testing period are killed and necropsied at the end of the observation. In order to increase the reproducibility of the study, all the experimental conditions and procedures should be standardized. Most importantly, the study must be conducted according to the good laboratory practices which are generally recognized (Wallace, 1989).

LD\textsubscript{50} is the value which is commonly used in acute toxicity studies. In this research project, it is also an index to define acute toxicity. But actually there are many other chemical constants like pH, pK\textsubscript{a}, melting point, etc., which are also available to define acute toxicity. Thus, there must be an idea that LD\textsubscript{50} is not the only index available to define acute toxicity. As the first defense line, the acute toxicity test in different conditions and exposure routes is considered. This depends on the sound scientific factors, which always vary from one chemical to another. In this research, two
exposure routes, i.g. and i.p. were performed in the NIH mouse (Gad and Chengelis, 1998).

10.3 MATERIALS AND METHODS

10.3.1 Materials

10.3.1.1 Materials

Tween-80 was from Sigma Chemical Co. USA. Deionized water was generated from a Milli-Q water system (Millipore, Bedford, MA, USA). NIH mice of 18~22 grams with SPF grade were from Guangzhou University of Traditional Chinese Medicine.

10.3.1.2 LIG Preparation

LIG was extracted, separated and purified from Radix Angelica sinensis in our laboratory, with batch number: 050118. Purified LIG was identified by electron impact ionization (EI) MS, H\(^1\) NMR and C\(^{13}\) NMR spectrometric techniques. The purity was found to be > 98% based on the percentage of total peak area by GC assay.

10.3.2 Methods
10.3.2.1 Animal and Drug Preparation

NIH mice of 18～22 grams with SPF grade were obtained from Guangzhou University of Traditional Chinese Medicine. They were kept in an environmentally controlled breeding room of temperature at 24～25℃, humidity: 40～60% with 12h dark/light cycle. Food and water were freely available. Before the test, the mice were fasted for 12h.

10.3.2.2 LIG Treatment Schedule

Dosages were generally based on the body weight of the animals. This was expressed in a weight form of the LIG suspension per kilogram according to animals’ weight. Before the application, LIG was diluted into 2 and 8 mg/ml with 3% Tween-80. NIH mice were used in the acute toxicity test to find out the LD₅₀ using a variety of dosages (from 200mg/kg to 1400mg/kg) by i.g. and i.p.. The i.g. groups of animals were fed with LIG with feeding needles attached to a syringe. The i.p. groups were injected with sterile injection needles (Gad and Chengelis, 1998).

10.3.2.3 Estimation of Acute Toxicity

Symptoms and time were recorded starting from injection to death. The observational index included appearance, activity, psychosis, urinary and fecal discharges, breath,
secretion of nasal, eyes and mouth, weight and death right after administering LIG. Dead mice should be dissected and have pathological organs check.

Observation was done for 2 weeks, and animals were weighted and recorded at a 7-day interval in the experiment. If animals survived after the two weeks, they were killed by breaking their backbone. Then, they were dissected to see if there is any abnormality. If yes, abnormality was recorded. The mortality rate of different dosage groups was calculated. Then, the software LD$_{50}$ was used, with the method of Bliss to find out the value of LD$_{50}$ for the two tests – i.g. and i.p..

10.4 RESULTS (Table 10-1, Table 10-2)

According to the method of acute toxicity test, a series of mice were administrated with variety dosages (from 200mg/kg to 1400mg/kg) by i.g. and i.p.. The appearance, activity, psychosis, urinary, fecal discharges and death of mice were observed immediately after administering LIG.

The time of death and the symptoms after drug injections of the animals were recorded. The results were showed in Table 10-1 and Table 10-2. The symptoms of the animals included cyanosis (bluish appearance of ails and foot pads), somnolence (drowsy but aroused by prodding and then resumed normal activities), tremors (trembling and quivering of limbs or entire body) and tonic convulsion (persistent contraction of muscles with rigid extension of hind limbs). The NIH mouse was dissected after death to see if there had any abnormality within the internal organs. But no observable abnormality was found.
The result of acute toxicity test was based on the Bliss method of the software LD_{50} with basal mortality setting to 0%. After using the software LD_{50} to calculate for different groups, it was found that the LD_{50} of the i.p. group was 259.04mg/kg, and the i.g. group was 649.71 mg/kg.

10.5 DISCUSSION

The acute toxicity of LIG was investigated in this research. LIG was assessed by gas chromatography to ensure it has a purity level of no less than 98%. For acute toxicity test, it was recommended to use pure compound. Since pure LIG required complicated purification process which might greatly increase the experimental cost, samples with no less than 98% purity were used. This can not only save the experimental cost but also be acceptable to acute toxicity testing in the primary pharmacological studies. (Zhao et al. 2003, Lo et al. 1995)

LIG is a new compound in pharmacology and toxicology, so the acute toxicity test is required to assess the intrinsic toxicity. Besides, the acute toxicity test of LIG can also provide information to the selection and design of dosage levels for future studies. In addition, the symptoms and the result of dissection of NIH mouse were also observed to see if LIG would cause any effects on the non-targeted organs of the animals. From the recorded symptoms, it was found that LIG caused effect in the central nervous system (CNS) and the neuromuscular tissues.
The observation period of acute toxicity test in this project was set to be 14 days. This was done to ensure the delayed acute effects could also be detected. For the dosage, five doses were set in both the i.p. and i.g. groups. All chemicals would be toxic if very large dose is given. The acute toxicity study should be conducted at a reasonable high dose to demonstrate lethality and toxicity. Extremely high dose may cause gastrointestinal blockage which is not related to the intrinsic characteristics of the test substance. Thus, the dosages used in this research are all within a test limit of 2.0g/kg (Wallace, 1989).

By using the LD$_{50}$ software, the LD$_{50}$ of the two groups of animals, subjected to either i.g. or i.p., were found out. The LD$_{50}$ in both i.g. and i.p. group gave an idea and information to the risk assessment of acute exposure and the dose level for further studies in the future. From LD$_{50}$ of the two groups, it could be concluded that the toxicity of tested LIG is of a low toxicity level.
Table 10-1. The result of intraperitoneal injection (i.p.) group

<table>
<thead>
<tr>
<th>Dosage (mg/kg)</th>
<th>Log(dosage)</th>
<th>Total no. of animals</th>
<th>Total no. of death</th>
<th>Mortality %</th>
<th>Porbit</th>
</tr>
</thead>
<tbody>
<tr>
<td>500</td>
<td>2.69897</td>
<td>14</td>
<td>13</td>
<td>92.86</td>
<td>6.47</td>
</tr>
<tr>
<td>400</td>
<td>2.60206</td>
<td>15</td>
<td>12</td>
<td>80.00</td>
<td>5.84</td>
</tr>
<tr>
<td>320</td>
<td>2.50515</td>
<td>13</td>
<td>6</td>
<td>46.15</td>
<td>4.90</td>
</tr>
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<td>256</td>
<td>2.40824</td>
<td>14</td>
<td>9</td>
<td>64.29</td>
<td>5.37</td>
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<tr>
<td>204</td>
<td>2.30963</td>
<td>13</td>
<td>4</td>
<td>30.77</td>
<td>4.50</td>
</tr>
</tbody>
</table>

After using the software LD$_{50}$ to find out the LD$_{50}$ of i.p. group, it found out that the LD$_{50}$ was 259.04 mg/kg ($A=-5.94967; B=4.537039$).

Table 10-2. The result of intragastrical injection (i.g.) group

<table>
<thead>
<tr>
<th>Dosage (mg/kg)</th>
<th>Log(dosage)</th>
<th>Total no. of animals</th>
<th>Total no. of death</th>
<th>Mortality %</th>
<th>Porbit</th>
</tr>
</thead>
<tbody>
<tr>
<td>2000</td>
<td>3.30103</td>
<td>15</td>
<td>15</td>
<td>100.00</td>
<td>7.13</td>
</tr>
<tr>
<td>1400</td>
<td>3.146128</td>
<td>14</td>
<td>14</td>
<td>100.00</td>
<td>7.10</td>
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<tr>
<td>980</td>
<td>2.991226</td>
<td>13</td>
<td>10</td>
<td>76.92</td>
<td>5.74</td>
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<tr>
<td>686</td>
<td>2.836324</td>
<td>14</td>
<td>10</td>
<td>71.43</td>
<td>5.57</td>
</tr>
<tr>
<td>480</td>
<td>2.681241</td>
<td>13</td>
<td>2</td>
<td>15.38</td>
<td>3.98</td>
</tr>
</tbody>
</table>

After using the software LD$_{50}$ to find out the LD$_{50}$ of i.g. group, it found out that the LD$_{50}$ was 649.71 mg/kg ($A=-9.21937; B=5.055378$).
CHAPTER 11

GENERAL DISCUSSION

Ligustilide (LIG) is the active constituent of *Angelica sinensis* and *Ligusticum chuanxiong*. The content of LIG in *Angelica sinensis* and *Ligusticum chuanxiong* was compared by the methods of TLC, HPLC, GC, GC-MS in this study, and it was found that there was a higher content of LIG and less other similar compound in *Angelica sinensis*. So *Angelica sinensis* was the ideal raw material to extract LIG. Here LIG was from *Angelica sinensis* volatile oil (ASVO). And the extraction process for ASVO was investigated with comparisons of steam distillation, ethanol extraction and supercritical fluid extraction (SFE). All these comparison ways were very common in extracting volatile oil and low polar components. Regarding LIG was not only a low polar component, but also an thermal instable compound, SFE was the best method to extract LIG from *Angelica sinensis* ---the SFE method with extraction and distillation features could be used in a low temperature without any remaining solvent, and it required a short operation time with high efficiency, also was applicable for the extraction of thermosensitive components, and could prevent from oxygenating and degrading. So SFE was the best method to extract ASVO from *Angelica sinensis*. The uniform design was further used to optimize the extraction process of SFE. And the optimum conditions for SFE were: 50°C, 5500Psi, 10% ethanol modifier and 4 hrs of
It was the first report on the separation of LIG by using Decompression column chromatography (DCC), also the first report to realize the large-scale production of LIG. It had broken the technical bottleneck of the industrialized separation of LIG. Gravity silica gel column chromatography was a commonly used method for LIG isolation. However, it was time-consuming, reagents-consuming, with a rather low efficiency. So a comparison study to other ways was carried out in present study by using pressure reducing distillation (PRD), pressurized column chromatography (PCC), and decompression column chromatography (DCC) respectively. Based on the characteristics of LIG, they had different efficacy of separation. PRD separated substances on different boiling points and sharply reduced the separation time, but it had a low purity of LIG, because some other components had similar boiling points. PCC was less time-consuming than gravity silica gel column chromatography and had a good efficacy of separation, but it only served for experimental level preparation due to its complicated operation, time-consuming and reagents-consuming procedures. DCC was the most effective and convenient method for LIG separation with an approximate 98% purity for the output. This study could enlarge LIG separation scale to a pilot scale, as the results showed a purity of 98.1% for output, indicating that this kind of separation method was suitable for large-scale production. DCC for LIG separation has several advantages. Firstly, there are its simple operation, simple equipments, rapid separation speed and high recovery rate. Then, it has the lowest extracting time.
requirement for materials and solvents and can save costs as well as contribute to environmental protection. Also, there is no time restriction, e.g., it could be stopped at anytime without affecting the isolation effect. Lastly, its separation scale could be enlarged to make it completely appropriate for industrialized large-scale production. Thus, this study has broken the technical bottleneck of the industrialized separation of LIG. It is the first report on the separation of LIG by using such kind of method, also the first report to realize the large-scale production of LIG. We had applied the invention patent in China in 2005. Now the patent has been granted for this new isolation method.

The peak area normalization method of chromatogram was used for the purity assay of LIG. Under certain chromatographic conditions, the concentration of the components in mobile phase showed a positive correlation with the peak area or peak height of the response signals of the detectors. As the size of the peak area was not subject to influences from the operating conditions, such as column temperature, flow velocity of mobile phase, and sample introduction rate, the peak area was more appropriate for a parameter of quantitative analysis. Taking the sum of all the components as 100%, and their related response signals as quantitative parameters, the normalization method could work out the results easily and accurately, especially for those liquid samples whose volume were difficult to measure accurately because of their small amount of sample introduction. HPLC, GC and GC-MS used in present experiment were equipped with an electric integrating instrument to accurately
measure the peak area, so the report from detecting system was directly based on the percentage of the total peak area by every analysis method. In this experiment, the result of the percentage of total peak area showed the content of LIG was 98.4% by GC-MS assay, while 98.3% by GC and 98.6% by HPLC, respectively. There was a slightly difference among the results of the three assaying methods because of systematic errors caused by the different apparatuses, which wouldn’t affect the final conclusion. The results of LIG content indicated that the purity of the prepared LIG was over 98% and could be used as the reference of LIG as well as raw materials for the new drug development of LIG.

It was the first completely report that physical and chemical property of LIG was carried out in this experiment. LIG is a slightly yellow oily liquid with a fresh sweet scent and gives a little astringent taste and tongue-numbing feeling. The measured density of LIG was 0.979 ±0.005 g/ml, which was slightly lower than water. And the water solubility of LIG was 0.004±0.0002% (w/w), also it was very soluble in organic solvents such as ethanol, methanol, acetic ether, ethyl acetate, petroleum ether, etc. The measured normal boiling point of LIG was 296-299°C, and the vacuum boiling point was 166-169°C/6mmHg. It was revealed that LIG was a highly liposoluble substance, with a lipid-water distribution coefficient of 3790±185. These results of LIG properties could provide a good foundation for the development of LIG.

There was the first completely report all factors affecting the stability of LIG.
Comparison studies were conducted to explore the factors affecting the stability of LIG. Temperature, light and oxygen all turned out to have an obvious influence to LIG’s stability. Thus the protection from high temperature, light and oxygen is needed as much as possible for the conservation of LIG. Since LIG is also extremely unstable in acidic and reductant conditions and could react with acid and reductant to produce new compounds, therefore, LIG must be kept away from acid and reductant in the pharmaceutical preparation and application. In one word, oxidation, hydrolysis, and photodegradation were shown to be the major degradation routes of LIG. The characteristic of LIG had specially influenced its own application and development. Regarding the 8 common metal ions, it had no obvious effect to LIG’s stability, so there was no need to pay special attention to this aspect. Meanwhile, Antioxidant could improve the stability of LIG, and the use of Vitamin E was a great help to minimize the decomposition of LIG. All these had laid a good foundation for improving LIG’s stability and designing new preparations, and the results could be further applied to other cases.

The high stable LIG reference was the first prepared with the suitable stabilizer. In this study, in order to get a suitable stabilizer to improve the chemical stability of LIG, factors that may affect its stability were investigated. To prepare the LIG reference, there were specific requirements for the added stabilizer. It must have no influence on the quality of LIG and no inlet of other ectogenic substances. And the stabilizer must have a great improvement on the stability of LIG in severe conditions, which are also
critical to the successful development of LIG reference. Finally suitable stabilizer for LIG was provided. Also it was proved to have an obvious protective effect on LIG against high temperature.

In the study on the shelf life of LIG reference, since the LIG stability is affected by the factors of oxygen, temperature, light etc., the degradation reaction mechanisms must be different at different times and temperatures, thus leading to different reaction order and activation energy. Accordingly, we applied the initial average speed (IAS) method, which is not influenced by reaction order or activation energy, to predict the shelf life preliminarily. This method is proved to be convenient and fast with a better accuracy. Also the elementary study was carried out on the prepared LIG reference with the accelerated testing. Then the shelf life of the LIG purity was found to be decreased by 2% at room temperature of 25°C after 8 months, indicating that the prepared LIG reference had a good stability and could meet the required standard. The temperature, however, still tended to be a comparative evident influential factor to LIG’s stability. For normal storage, the replacement of LIG in a hypothermic refrigerator is still recommended, as a longer shelf life could be obtained in this way.

The degradation reaction of LIG is a complicated multiple reaction. Oxidation, hydrolysis and photo-degradation are the major degradation routes of it. And the degradation reaction mechanism is different in different storage conditions. To innovate a new drug, it is necessary to study the properties of their degradation
products. The determination of degradation products and the related products plays an important role in the research and even in the development of a new drug. In this experiment, investigations applying GC-MS were also performed on the degradation productions of LIG, and the structure of the degradation compounds was obtained. Then the basic degradation pathway of LIG was deducted. It would benefit further work on the pharmaceutical preparations and clinical application of LIG.

LIG was obtained successfully with chemical synthesis. The key step in LIG synthesis is the Birch reaction of 3-butylidene-phthalide. In this experiment, phthalic anhydride was used as raw material, and then butyl from butyl lithium was adopted as introductive functional group. 3-butylidene-phthalide was finally obtained through the reaction after P-TsOH and Benzene was added. It was ever reported (Li et al., 1995) before, however, the report, in which Natrium was used as reactant, didn’t have any LIG made out till the very end. Now in this experiment, after several trial and error, finally Lithium was selected to replace Natrium and then LIG was obtained successfully. Even though, the yield rate was still very low at only about 3%, but the condition of synthesis was pretty critical, which have brought great inconvenience to its industrialization of manufacture. We had ever tried many ways of synthesis, but only failed to find a better way to make LIG more easily with much higher yield rate. Moreover, LIG itself still had very special chemical structure as well as high instability, which had made its synthesis become difficult. Thus, the industrialization of manufacturing LIG seemed to be unpractical at present.
It was the first report that LIG CD inclusion complexes were prepared successfully and had very good stability. Hydroxylpropyl-β-cyclodextrin (HP-β-CD) was adopted as the host material for LIG inclusion complex. The binding of LIG to HP-β-CD is dependent on the strength of the van der Waals’ force between them. If the van der Waals’ force is weak, the inclusion complexes will not be stable and thus may lead to a low yield of inclusion complexes (Shehatta, 2002). We are unable to control the strength of the van der Waals force among the assigned molecules, however, the performance of the inclusion process can be affected by other factors, such as the process method of preparing the inclusion complexes and the proportion of guest molecule to host molecule. Thus, several methods were used to prepare the inclusion complexes, and high speed stirring was the most suitable preparation method for LIG-HP-β-CD mixture solution in laboratory condition because the least re-suspend droplet of LIG occurred in the samples prepared by this method. Also we had tried different proportions of LIG to HP-β-CD to test which proportion could give the highest inclusion ratio, and the best one was turned out to be 1:1 as molecular ratio.

LIG itself is aqueous-insoluble, but when it is included by HP-β-CD, it become aqueous-soluble. Thus the UV absorbance of LIG can't be displayed because it can’t be dissolved in water, while the situation will be quite opposite if it is with inclusion. As a control, when LIG was in starch, the UV absorbance was nearly 0. And the UV absorbance of the LIG CD inclusion complexes increase with the increase of the
amount of included LIG until the inclusion achieve saturation, during which the UV absorbance will be the maximal value. In this experiment, however, it might not reach this ideal maximum value. The theoretical maximal amount of LIG at saturation can be calculated by the linear regression equation of the absorption curve, as the absorption value of LIG at saturation is known. This is a new way to detect and calculate the maximal amount of drug for the inclusion of HP-β-CD.

The stability of LIG included by HP-β-CD was greatly enhanced under the environment of high temperature, strong light and high humidity, while the LIG un-included by HP-β-CD was degraded largely and quickly. This suggested that it be completely viable to improve the stability of LIG inclusion by HP-β-CD. Besides, we didn’t remove the oxygen from the samples when the accelerating experiment was carried out, which meant the effect of air existed in the accelerating experiment. But, actually, the influence of oxygen to the stability of LIG could be eliminated if proper oxygen-excluding application was undertaken, thus the LIG’s stability in LIG CD inclusion complexes would be improved more greatly. The LIG CD inclusion complexes prepared according to the preparation process not only increased the stability of LIG, but also made it dissolved in water fully, showing that the inclusion preparation of HP-β-CD was suitable for LIG. Meanwhile, the liquid LIG was transformed into solid so that it could be used as the raw drug materials for oral tablets, powder for injection and so on, which would surely widen the application of LIG preparations remarkably.
It was the first report that LIG micro-emulsion (ME) was prepared successfully and had very good stability. ME is a mixture composed of multi-components, with at least three, usually four or five components. Single, double or triple phase can be obtained by changing the parameters of system. Phase diagram is the basic tool for the study of ME. The determination of the phase border is very important in the study of ME. The formulation design of ME relies on the proper selection and ration of components to enable the fitness of the components for pharmaceutical purpose and ME formation in a wide range. In the preparation of LIG ME, single factor tests were adopted to determine the best emulsifier, the best oil phase, the ratio of emulsifier phase and oil phase. The pseudo-ternary phase diagram for the emulsifier phase, oil phase and aqueous phase was made to ascertain the optimal forming region of ME. And LIG ME was prepared by adopting soybean phosphatide as an emulsifier, ethanol as a co-emulsifier, and olive oil as an oil phase. Also the optimum proportion and method of LIG ME preparation was obtained with a series of the preparation process experiments. The stability of LIG ME was studied by accelerated testing, and it was found that the stability of LIG would increase greatly upon the optimum inclusion ratio and well prepared process. And the method of LIG ME could establish a solid foundation for LIG to develop new pharmaceutical preparations.

LIG has a low level toxicity and could be complied with the safety requirement of new medicine. LIG is a new compound in pharmacology and toxicology, so the acute
toxicity test is required to assess the intrinsic toxicity. In this study, acute toxicity test used the index LD$_{50}$ to define the intrinsic acute toxicity of the high pure LIG with NIH mouse. The LD$_{50}$ was calculated by software LD$_{50}$ with the method Bliss which adopted the mortality rate. The result indicated that LIG was safe to use as a drug because the LD$_{50}$ of intraperitoneal injection (i.p.) and intragastrical injection (i.g.) group was 259.04 mg/kg and 649.71 mg/kg respectively. From the results, it could be concluded that the toxicity of LIG is of a low level. Besides, the acute toxicity test of LIG can also provide information to the selection and design of dosage levels for future studies. In addition, the symptoms and the result of dissection of NIH mouse were also observed to see if LIG would cause any effects on the non-targeted organs of the animals. From the recorded symptoms, it was found that LIG caused effect in the central nervous system (CNS) and the neuromuscular tissues.

All above studies could establish a solid foundation for LIG to develop a new medicine in first category.
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