Simultaneous determination of saikosaponins in Bupleurum Radix from different locations by high performance liquid chromatography- charged aerosol detection (HPLC-CAD) method and its immunomodulation effects on mouse splenocytes

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A high performance liquid chromatography (HPLC)-Charged Aerosol Detection (CAD) was employed to simultaneously determine saikosaponins which include saikosaponin a (SSa), saikosaponin b2 (SSb2) and saikosaponin d (SSd) in Bupleurum Radix from different locations. Meanwhile, MTT assay was used to compare the proliferation effect of SSa, SS and SSb2 on mouse splenocytes, and enzyme linked immunosorbent assay (ELISA) method was used to determined cytokines which include interleukin (IL)-4, IL-10, interferon (IFN)-γ, TNF-α. The results show that there existed significant differences of the three saikosaponins from different locations, and SSa, SSD, and SSb2 all presented immunomodulation effects on mouse splenocytes via promoting splenocyte proliferation and regulating the balance of Th1/Th2 factors.

Key words: HPLC-CAD, Bupleurum Radix, saikosaponin a (SSa), saikosaponin b2 (SSb2), saikosaponin d (SSd).

INTRODUCTION

Bupleurum Radix, a well-known traditional Chinese medicine (TCM), was used for the treatment of common cold with fever, influenza, hepatitis, malaria and menoxenia. Bupleurum chinense DC. and Bupleurum scorzoneri folium Willd. recorded in the Chinese Pharmacopoeia are officially regarded as standard medicinal plants (The Pharmacopoeia Commission of PRC, 2010). Also, Bupleurum Radix was widely distributed in China other than Hainan province. Bupleurum chinense DC. grows in Northern China, and Bupleurum scorzoneri folium Willd. is mainly distributed in Southern China, of which 44 species, 17 varieties have been reported in China and nearly half of them are native species (Shan and She, 1979). Many compounds were found in Bupleurum including phytocholesterols and volatile oil, polysaccharides, saikosaponin a (SSa), saikosaponin b2 (SSb2), saikosaponin d (SSd) and so on (Shi et al, 2002).Recently, pharmacology studies indicate that the saikosaponins (SS) have many pharmacological effects such as, relieving fever, conscious sedation, anti-inflammatory, immunity regulating, antivirus, anti-liver fibrosis, anti-tumor, relieving pain, anti-biosis, protecting a liver and anti-nephritis (Zhu et al, 2002; Huang and Sun, 2010). Some studies report that SS presents specific and

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nonspecific immunity functions, and induces the aggregation of peritoneal macrophage, activates and stimulates the T lymphocyte and B lymphocyte to participate in the immunomodulation, and improves the activity of cell surface receptor on the of macrophage and changes the ultrastructure in cells so as to enhance their activities (Wei, 2005; Mohamed and Michael, 2011), and the above studies mainly concentrated on SSa and SSd, but the study about SSb2 has not been reported until now.

Therefore, it is necessary to investigate their discrimination and develop a reasonable approach to control their quality. In recent years, the employment of HPLC-UV (Li et al, 2005; Mahmood et al, 2011; Emira et al, 2011) and ELSD (Pu et al, 2010) method to determine SSa, SSb2 and SSd has been reported. But little information about the determination of saikosaponin in Bupleurum Radix using HPLC-CAD method has been published. In this study, HPLC-CAD method was first employed to simultaneously determine SSa, SSb2, SSd, the main biomarkers in Bupleurum Radix from different locations. In addition, to compare immunomodulation effects of the saikosaponins in the Bupleurum Radix, MTT assay was used to test the effects of different concentrations of SSa, SSd, and SSb2 on the mouse splenocyte proliferation, and ELISA method was applied to determine in vitro adjusting lymphocyte factors, such as IL-4, IL-10, IFN-γ, TNF-α.

MATERIALS AND METHODS

Plant material

Bupleurum was collected from thirteen different locations in Liaoning, Inner Mongolia, Jilin, Heilongjiang, Shanxi, Hebei, Anhui and so on. Voucher specimens were maintained at Liaoning University of Traditional Chinese Medicine, China. They were identified by Professor Yanjun Zhai in School of Pharmacy, Liaoning University of Traditional Chinese Medicine.

Chemicals

The standard substances, SSa, SSb2 and SSd were provided by VKEIQI biological products Co., Ltd (Sichuan, China). HPLC-grade acetonitrile was purchased from Damao chemical reagent plant (Tianjin, China) and the water used in all experiments was purified by a Milli-Q® Ultrapure Water System (Millipore, MA, USA). RPMI-1640 culture solution was purchased from Solarbio, USA, Thiazole blue (MTT) and sword bean protein A (ConA) from Sigma, USA, Red blood cell disruption solution from Solarbio, USA, and ELISA kit from R&D, USA. All other chemicals were of analytical reagent grade purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China).

Animals

All animal studies were performed according to the Guidelines for the Care and Use of Laboratory Animals that was approved by the Committee of Ethics of Animal Experimentation of Liaoning University of TCM. BA CL mice were purchased from the Experimental Animal Center of Dalian Medical University (qualified card number Liaoning 2008-0002). They were kept in an environmentally controlled breeding room for one week before the experiments and fed with standard laboratory food and water.

Instrument and chromatographic conditions

CO2 incubator was purchased from SANYO MCO-175M, Japan. Clean Benches from ZHUJ-1112B, China. Multiskan Mk3 microplate reader from Thermo, China, and NIB-100 inverted biological microscope from NOVEL China. HPLC analysis was carried out on Shimadzu LC-20A high performance liquid chromatography (LC-20 pump, SIL-20 autosampler, A CTO-20 constant temperature column oven, A CBM-20 TSC, LC Solution work station), incorporating an electrical aerosol detector (CAD). The analytes were determined at 20°C on an analytical column Waters C18 (4.6 mm × 150 mm, 5 μm). The mobile phase consisted of the solvent acetonitrile-water gradient elution (30:70-50:50). The mobile phase was passed under vacuum through a 0.45 μm membrane filter before use. The analysis was carried out at a flow rate of 1 ml/min. Injection volume was 10 μl.

Sample preparation

To the conical flask with lid, 1 g of the dried and powdered Bupleurum were extracted with 25 ml of a mixture of methanol-ammonia solution (95:5, v/v) in an ultrasonic bath for 30 min at 30°C. The supernatants were collected and then the residue of Bupleurum was washed with 20 ml methanol twice. Both of these fractions were combined and evaporated to dry. Finally, the residue was reconstituted by 10 ml methanol and supernatants were filtered with 0.45 μm membrane filter to obtain the filtered solution. An aliquot (10 μl) of filtrate was injected into the HPLC system.

Method validation

Linearity, LLOQ and LOD

Stock standard solutions of SSa, SSb2 and SSd were prepared with methanol. The three calibration curves in the concentration ranges of SSa, SSb2 and SSd were 0.088-8.8, 0.044-4.4 and 0.088-8.8 μg/ml, respectively. The calibration curve was constructed using six different concentrations by plotting the peak area versus the nominal concentration. LOD and LOQ were determined by stepwise dilution of the QC sample at low concentration level using a signal-to-noise ratio of 3 and 10, respectively.

Precision and accuracy

The precision and accuracy of the method were evaluated with QC samples at low, middle and high three concentrations and using five replicates on three consecutive days. The intra- and inter-assay precisions were assessed by determining the QC samples at three concentration levels of each compound. For the intra-day validation, five replicates of the QC plasma samples were analyzed on the same day, while the inter-day values were carried out over three consecutive days. The accepted criteria for each QC sample were that the precision (RSD) and accuracy (RE) should not exceed 15%, except at the LOQ where it should not exceed 20%.

Recovery

The recovery was determined by adding known amount of the standard substances of SSa, SSb2 and SSd, approximately one times the levels detected in unspiked samples prior to extraction, and
these spiked samples were prepared as described in "Sample preparation". The total amount subtracted the content in unspiked sample was divided by the added amount of the standard substance, and the extraction recovery was calculated as percentage.

### Stability

The stabilities of SSa, SSb2 and SSd in the methanol were investigated by comparing the peak area of the three saikosaponins at 3, 6 and 12 with that at 0 h, that is, the stabilities of three compounds in the solution of methanol were investigated during the storing period of 0-12 h.

### Preparation of different concentration solutions of SSa, SSd and SSb2

The different concentration (Figure 2) solutions of SSa, SSd and SSb2 were respectively prepared by adding RPMI-1640 culture medium solution then filtrated through 0.22 µm millipore filtration membrane before use.

### Preparation of splenocytes (Li, 2006)

BACL mice (n = 6) were soaked in 75% ethanol for 3 min after sacrifice, and the spleen was cut off under the sterile conditions, washed successively with PBS containing different concentrations of the double antibiotics including penicillin and streptomycin, levigated, filtrated through 0.22 µm millipore to obtain cell suspension, added 3-5 times of red blood cell disruption solution, transferred into a centrifuge tube, shaken for 5 min at room temperature, centrifuged at 1200 rpm for 10 min, and the white precipitation was washed with PBS for twice, with culture solution for 1 time, centrifuged at 1200 rpm for 10 min after each wash. Finally, the white precipitation was respectively added the calf serum and RPMI-1640 culture solution containing double antibiotics (100 U/ml), vortexed, dyed by trypan blue and then counted. The cell concentration will be adjusted based on the demand of the test before use.

### MTT assay

The above spleen cells (1 × 10⁶/ml) were seeded in each well of 96-well plates, 100 µl for each well, and maintained at 37°C with 5% CO₂ under aseptic conditions for 48 h, added ConA whose final concentration in the culture solution was 5 µg/ml, and 20 µl different concentration of drugs, added with culture solution up to the volume of 200 µl, incubated for 48 h, and then MTT (20 µl/well) was added to each well, and culture was terminated after incubated at 37°C for 4 h, centrifuged at 1000 rpm for 5 min to remove the supernatant. After that, 150 µL DMSO was added in each well, shocked for 10 min, and then the plates were read using the microplate reader at a wavelength of 492 nm. All of the assays including blank control ConA(+) and negative control ConA(-) were performed at least in triplicate.

### ELISA assay

The concentration of spleen cells was adjusted to 1 × 10⁷/ml, and then the cells were seeded in each well of 24-well plates, 100 µl for each, and maintained at 37°C with 5% CO₂ under aseptic conditions for 48 h. Added was ConA whose final concentration in the culture solution was 5 µg/ml and 20 µl of the three drugs screened by above MTT assay, cultivated for 48 h, and then the supernatants were assayed to determine IL-4, IL-10, IFN-γ, TNF-α, and OD values based on the instruction of ELISA kit at 450 nm. All of the assays including blank control ConA(+) and negative control ConA(-) were performed at least in triplicate.

### Statistical analysis

All results were confirmed in at least three separate experiments. Data are shown as mean ± standard deviation and were analyzed by one-factor analysis of variance. A value of p<0.05 was considered significant.

### RESULTS AND DISCUSSION

#### HPLC analysis

The saikosaponin is the main ingredient of Bupleurum including SSa, SSb, SSd, in which many pharmacological studies about SSa and SSd have been reported, and SSb were transformed from SSa and SSd in the extraction process (Harada, 1992), that is, SSb1, SSb2 were respectively transformed from SSa and SSd. Therefore, the three saikosaponins are taken as the indexes for the determination except that SSb1 because which was unstable, additionally, its reference substances are not readily available.

The reflux method has been used to extract SSa and SSd, however, the contents of them is remarkably lower than that of ultrasonic, and the content of SSb2 slightly increased meaning that the transformation of saikosaponins occurred in the process of heating reflux. Therefore, the ultrasonic extraction was applied for sample preparation because the contents of SSa and SSd are higher than that of reflux method. In addition, a mixture of methanol-ammonia solution (95:5, v/v) was used to extract the sample because it can avoid saponins transformed (Fu et al., 2010).

Our attempts to use the method with isocratic elution for the determination of SSa, SSb2 and SSd were unsuccessful. The gradient elution method was therefore used for the separation of them. The maximum absorption determined in this experiment of SSa and SSd is at 204 nm and SSb2 is at 254 nm, respectively, in which 204 nm were close to the end absorption presenting low sensitivity, baseline drift when Bupleurum saponins were detected by HPLC with UV detection (Li et al., 2005) and ELSD detection (Pu et al., 2003). Therefore, an HPLC-CAD method was used to determine SSa, SSb2 and SSd to obtain a good separation, symmetric peaks and little interference chromatogram. Figure 1 shows typical chromatograms of the standard substances (A), and the SSa, SSb2 and SSd in Bupleurum Radix (B). The retention times of SSa, SSb2 and SSd were approximately 19.3, 21.0 and 30.9 min, respectively, and the total chromatographic run time was 40 min.
Method validation

Linearity, LLOQ and LOD

The evaluation of the linearity was performed with a six-point calibration curve over the concentration range of 0.088-8.8, 0.044-4.4 and 0.088-8.8 μg/ml for SSa, SSb2 and SSd, respectively. The calibration curve was constructed using six different concentrations by plotting the peak area versus the nominal concentration. The regression equations, coefficients and results of LOD and LOQ are shown in Table 1. Precision and accuracy of LOD and LOQ were within 15% after repeated analysis.

Precision and accuracy

The summaries of intra- and inter-day precision/accuracy at low, medium, and high concentrations of each analytes in plasma are listed in Table 2 indicating that the procedures described above were satisfactory with respect to both accuracy and precision.

Recovery

For a validation of the extraction recoveries of the three compounds, the analysis for each biomarker was carried out in six replicates. The results show that the mean extraction recoveries were acceptable ranged from 95.92% to 99.69%, suggesting that there was negligible loss during the leaves extraction.

Stability

The stock solutions of three compounds were found to be stable at room temperature over the time range of 0 to 12 h.
Figure 2. SS effects on the cytokines (a) TNF-α (b) IFN-γ (c) IL-4 (d) IL-10 and on splenocyte proliferation (e). * p < 0.05, ** p < 0.01, compared with blank group.

The stabilities of three compounds in the solution of methanol were investigated during the storing period of 0-12 h, RSD (%) of the area for three compounds were 4.59, 3.25 and 3.55%, respectively, suggesting that they were stable after the samples were treated as “Sample preparation” under the experimental conditions of the regularly analytical procedure.

Simultaneous determination of three saikosaponins

HPLC incorporating CAD method was employed to simultaneously determine three biomarkers in Bupleurum Radix from different locations. The dried and powdered leaves were treated as the “Sample preparation”. Table 3 shows the variations of three compounds in Bupleurum.
Table 1. Results of linearity, LOD, LOQ for SSa, SSb2 and SSd.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Regression equation</th>
<th>Coefficient (r)</th>
<th>Range of linearity (μg/ml)</th>
<th>LOD (mg/l)</th>
<th>LOQ (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSa</td>
<td>Y=526487.2X + 86905.0</td>
<td>0.9991</td>
<td>0.088-8.8</td>
<td>1.06</td>
<td>3.54</td>
</tr>
<tr>
<td>SSb2</td>
<td>Y=352021.9X + 67742.5</td>
<td>0.9990</td>
<td>0.044-4.4</td>
<td>0.604</td>
<td>2.01</td>
</tr>
<tr>
<td>SSd</td>
<td>Y=784478.2X + 131142.3</td>
<td>0.9985</td>
<td>0.088-8.8</td>
<td>0.371</td>
<td>1.24</td>
</tr>
</tbody>
</table>

Table 2. Contents of SSa, SSb2 and SSd at different locations (n = 5).

| Parameter   | Added C (mg/ml) | Intra-day | | | Inter-day | | |
|-------------|----------------|-----------| | | | | |
|             | Found C | RSD (%) | RE (%) | Found C | RSD (%) | RE (%) | |
| SSa         | 0.176   | 0.171±0.004 | 2.61 | 2.92 | 0.160±0.004 | 2.21 | 4.14 |
|             | 3.52    | 3.58±0.082 | 2.29 | 1.73 | 3.545±0.076 | 2.13 | 0.71 |
|             | 7.04    | 6.97±0.15 | 2.13 | 1.08 | 6.97±0.163 | 2.34 | 0.96 |
| SSb2        | 0.088   | 0.081±0.005 | 6.10 | 8.64 | 0.087±0.005 | 5.45 | 1.14 |
|             | 1.76    | 1.71±0.10 | 6.07 | 2.98 | 1.71±0.085 | 4.97 | 2.80 |
|             | 3.52    | 3.58±0.15 | 4.17 | 1.62 | 3.59±0.125 | 3.48 | 2.06 |
| SSd         | 0.176   | 0.169±0.005 | 3.13 | 4.14 | 0.170±0.004 | 2.37 | 3.53 |
|             | 3.52    | 3.55±0.09 | 2.74 | 0.79 | 3.66±0.096 | 2.62 | 3.88 |
|             | 7.04    | 6.99±0.15 | 2.18 | 0.74 | 7.02±0.164 | 2.33 | 0.30 |

Table 3. Precision and accuracy for the determination.

<table>
<thead>
<tr>
<th>Location</th>
<th>SSa</th>
<th>SSb2</th>
<th>SSd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hebei</td>
<td>3.14±0.27</td>
<td>0.142±0.012</td>
<td>3.24±0.25</td>
</tr>
<tr>
<td>Anguo of Hebei</td>
<td>4.09±0.35</td>
<td>0.183±0.009</td>
<td>4.10±0.56</td>
</tr>
<tr>
<td>Cultivation in Liaoning</td>
<td>7.25±0.68</td>
<td>0.142±0.013</td>
<td>8.86±0.78</td>
</tr>
<tr>
<td>Wild growing in Liaoning</td>
<td>4.08±0.52</td>
<td>0.542±0.049</td>
<td>4.19±0.21</td>
</tr>
<tr>
<td>Shandong</td>
<td>2.32±0.18</td>
<td>0.422±0.053</td>
<td>3.07±0.46</td>
</tr>
<tr>
<td>Jilin</td>
<td>3.46±0.45</td>
<td>0.078±0.098</td>
<td>4.55±0.39</td>
</tr>
<tr>
<td>Hubei</td>
<td>3.93±0.29</td>
<td>0.327±0.028</td>
<td>9.45±1.3</td>
</tr>
<tr>
<td>Cultivation in Inner Mongolia</td>
<td>4.58±0.37</td>
<td>0.419±0.056</td>
<td>11.8±1.09</td>
</tr>
<tr>
<td>Wild growing in Inner Mongolia</td>
<td>3.40±0.22</td>
<td>0.331±0.045</td>
<td>7.54±0.78</td>
</tr>
<tr>
<td>Gansu</td>
<td>3.77±0.33</td>
<td>0.242±0.019</td>
<td>7.79±0.84</td>
</tr>
<tr>
<td>Anhui</td>
<td>3.56±0.21</td>
<td>0.434±0.033</td>
<td>7.43±0.55</td>
</tr>
<tr>
<td>Shanxi</td>
<td>5.12±0.66</td>
<td>0.486±0.027</td>
<td>11.5±0.97</td>
</tr>
<tr>
<td>Heilongjiang</td>
<td>3.85±0.43</td>
<td>0.289±0.008</td>
<td>5.99±0.64</td>
</tr>
</tbody>
</table>

Radix from different locations, meaning that it existed a large difference in the quality of 13 batches from different Bupleurum Radix, and the commercially available herbs basically met to the standard of Chinese Pharmacopoeia (>3 mg/g). The contents of SSa and SSd were the highest respectively from Liaoning and Shanxi Cultivation. Conversely, the lowest of SSa and SSd occurred, respectively, in Hebei and Shandong. The results indicated that Bupleurum cultivars of Liaoning and Inner Mongolia have remarkable advantages, and this study provides a reference for the rationally exploiting and developing medicinal resources.
Assay on in vitro immunomodulation of saikosaponins

To judge and compare the immunomodulation effects of SSa, SSd and SSb2, MTT method was firstly applied to screen the immunocompetence of the saikosaponin via determining the influence of different concentrations of SSa, SSd and SSb2 on mouse splenocytes. Secondly, the in vitro immunomodulation factors, such as IL-4, IL-10, IFN-γ, TNF-α were detected by ELISA method. After achieving the effective concentration, each dosed group could enhance the transformative capacity of T lymphocyte induced by the ConA, and stimulated the proliferation of splenic lymphocyte, presented immunomodulation effects and good dose-effect relationship. At the same time, in vitro study showed that SS showed an adjusted effects on the cytokines released by T lymphocyte. In addition, the results indicated that SSb2 also had certain immunomodulation effect which existed no distinct differences compared to that of SSa but superior to that of SSd (Figure 2).

Under normally physiological conditions, Th1 cells and Th2 cells in an organism present dynamic balance which will evoke pathological reaction once losing the balance, and this is also called Th1/Th2 excursion which happen in many diseases such as microbial infection, tumor, autoimmunity disease, allergic reaction and transplant rejection (Xing and Zhang, 2002). Th1 and Th2 cells respectively, secrete IFN-γ and IL-4, and therefore discriminate of Th1 and Th2 cell must rely on the excretion of IFN-γ and IL-4. TNF-α being one of the members of tumor necrosis factor family, which play an important action in inflammatory reaction and apoptosis, is an important proinflammatory and immunomodulatory factors excreted dominantly from monocyte-macrophages and activated T lymphocyte, and this cause an extensive tissue damage. IL-10 is also called the inhibiting factor of cytokine, a multieffect factor presenting strong immunomodulation effects. Therefore, in the study, IL-4, IL-10, TNF-α and IFN-γ being the indices were selected to investigate the effect of SS on the contents of proinflammatory factors and anti-inflammatory factors of mouse splenocytes stimulated by ConA.

The immunomodulation effects of SS maybe adjust the abnormal function of cytokine network to allow Th1/Th2 recovering to the normal state. In our study, the contents of TNF-α, IFN-γ and IL-10 in the blank group were all higher than that in ConA (-) group, IL-4 in the blank lower than that in ConA group. The contents of TNF-α, IFN-γ in each dosed group were lower than that in the blank group, but still higher than those in ConA (-) group. When stimulated by ConA, the proinflammatory factor excreted by mouse splenocytes increased, SS could improve the above phenomenon and also showed some dose-effect relationship. In summary, SS can modulate the balance of Th1/Th2 factors and presents the immunomodulation effects via decreasing TNF-α, IFN-γ excretion and increasing IL-4, IL-10, furthermore, there was no remarkable difference of immunomodulation effects among SSb2, SSa and SSd.

Conclusion

A simple HPLC-CAD method was developed for the simultaneous determination the SSa, SSb2 and SSd in Bupleurum Radix from different locations. Meanwhile, the immunomodulation effects of the multi-saikosaponin in Bupleurum Radix were investigated. The results show that there existed significantly content differences in the three saikosaponins from different locations; and SSa, SSd and SSb2 all presented immunomodulation effects on mouse splenocytes.

REFERENCES