



New method of screening allergenic components from Shuanghuanglian injection: With RBL-2H3/CMC model online HPLC/MS system

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ABSTRACT

Shuanghuanglian (SHL) injection is a traditional Chinese medicine (TCM) injection widely used in China to treat influenza, tonsillitis, bronchitis, and faucitis. Adverse drug reactions (ADR) of SHL injection, majorly manifested as allergic reactions, were among the leading causes of death from TCM injection. In this study, an RBL-2H3/CMC online LC/MS system was established to screen and identify allergenic components in SHL injection, by which Baicalin was identified as a potential allergenic component. Allergenic activities of baicalin were investigated in RBL-2H3 cell degranulation and β -hexosaminidase release tests *in vitro*. Our results showed that baicalin dose-dependently induced RBL-2H3 cell degranulation and β -hexosaminidase release in the range of 0–50 μ g/ml. The RBL-2H3/CMC online LC/MS system developed in this study may potentially be used to screen allergenic components in other TCM injections.

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1. Introduction

Non-IgE mediated drug hypersensitivities, also referred to as pseudoallergic or anaphylactoid reactions, have clinical manifestations that are often indistinguishable from allergic reactions [1]. Anaphylactoid reactions, similar to IgE-mediated allergic reactions, cause mast cell degranulation and release of chemical mediators [2], which induce acute allergic responses such as smooth muscle contraction, vasodilation, and increased vascular permeability [3]. Mast cells play a central role in anaphylactoid reactions.

As a new dosage form of traditional Chinese medicine (TCM), traditional Chinese medicine injection (TCMI) has broad clinical application in China for the treatment of a variety of diseases including bacterial and viral infections, cardiovascular and cerebrovascular dysfunction, cancer, and rheumatoid arthritis [4,5]. However, adverse drug reactions (ADRs) of TCMI, including anaphylactic shock and fatal anaphylaxis, have been reported in more than a thousand clinical research and review articles in Chinese biomedical journals [6–8], which propelled the Chinese government to implement more stringent regulations on safety evaluation and quality control. In 2010, China Food and Drug

Administration published regulatory guidelines for safety evaluation of TCMI. Current methods for quick and accurate assessment of potential allergenic components in TCMI are inadequate, and new technologies are urgently needed to meet the increasing demand in this area.

Shuanghuanglian (SHL) injection is composed of certain proportion extracts of *Lonicera Japonica*, *radix scutellariae*, and *Forsythia*. It is recorded in Chinese Pharmacopoeia and approved for production by China Food and Drug Administration. SHL injection is clinically used to treat infections such as influenza, tonsillitis, bronchitis, and faucitis [9]. ADRs caused by SHL injection, majorly manifested as allergic reactions [12], were among the leading causes of death from TCMI [11]. The quality control of SHL injection, however, is still under resolve for its complex composition [10]. Particularly, no analytical method for screening and identification of allergenic components in SHL injection has been reported. In the present study, we aimed to develop an effective and practical method for allergen screening and identification in SHL injection.

The rat basophilic leukemia (RBL) cell line was developed in 1973 by inducing leukemia in rat fed with the chemical carcinogen, β -chloroethylamine [16]. RBL-2H3, a histamine-secreting clone was isolated from RBL sub-clone RBL-IV (HR+) in 1976 [17]. Even though RBL-2H3 cells are more like basophils than other histamine-releasing cell types in certain aspects [18], they have similar granular content to that of mast cells and are commonly

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employed as a prototypic and convenient model to study mast cell degranulation. Membrane receptors play a central role in mast cell degranulation [21,22]. Like in mast cells and basophils, FcεRI receptor cross-linking in RBL-2H3 cells activates degranulation and release of multiple mediators such as histamine, serotonin, and β-hexosaminidase [19,20]. In present study, we developed a chromatographic method using columns loaded with RBL-2H3 cell membrane to screen potential allergens in SHL injection.

Cell membrane chromatography (CMC) has been demonstrated as an effective method to screen target components in complex systems [23–25]. Meanwhile, liquid chromatography tandem mass spectrum (LC/MS) has been widely used in life science to analyze complex samples since 1990 [26,27]. In this study, RBL-2H3/CMC column chromatography was combined with LC/MS to build a two-dimensional analytical system for the isolation and identification of potential allergenic components in SHL injection (Fig. 1). Allergenic activities of the identified component were subsequently assessed interests measuring cell degranulation and β-hexosaminidase release [28,29].

2. Experimental

2.1. Reagents and chemicals

Silica gel (ZEX-II, 5 μm, 200 Å) was obtained from Qingdao Meigao Chemical Co., Ltd. (Qingdao, China). Baicalin (≥98%, lot#: MUST-11101403) and quercetin (≥98%, lot#: MUST-12072505) were purchased from Chengdu Must Bio-tech. CO., LTD. (Chengdu, China). SHL injection (lot#: 1007220) was provided by Second Chinese Medicine Factory of Harbin Pharm Group CO., LTD. (Harbin, China). HPLC grade methanol and acetonitrile were from SK Chemicals Co., Inc. (Ulsan, Korea). All aqueous solutions were prepared using ultrapure water produced by MK-459 Millipore Milli-Q Plus ultra-pure water system.

2.2. Instrument configuration and chromatographic conditions

The two dimensional analytical system was build with a DGU-20A₃ degasser, three LC-20AD pumps, an SIL-20A autosampler, a CTO-20AC column oven, an SPD-M20A diode array detector (DAD), an SPD-20A UV/Vis detector (UV), an LC/MS-2010EV mass spectrometer, and a LC/MS solution work station (Shimadzu Corporation, Kyoto, Japan). The system was controlled by a VICIAG 10G-0911V 10 port 2-position valve (Valco Instrument Co. Inc., Houston, USA). Two Shim-pack VP-ODS pre-columns (10 mm × 2 mm i.d., 5 μm, Shimadzu Corporation, Kyoto, Japan) were used as enrichment columns. An RBL-2H3/CMC column (10 mm × 2 mm i.d., 5 μm) packed using a RPL-10ZD column loading machine (Dalian Replete Science and Technology Co., Ltd., Dalian, China) was used as the first dimensional column, and a Shimadzu Shim-pack VP-ODS column (150 mm × 4.6 mm i.d., 5 μm, Kyoto, Japan) was used as the second dimensional column. The preparation of RBL-2H3/CMC column was described later in Section 2.5.

The RBL-2H3/CMC column was thermostated at 37 °C and run with mobile phase of water at a flow rate of 0.2 ml/min. The VP-ODS column was also thermostated at 37 °C and eluted with a gradient of 0.5% acetic acid aqueous solution (v/v) and acetonitrile at a flow rate of 1 ml/min. Percent acetonitrile started at 0% and increased linearly to 40% over a duration of 130 min. Quercetin and baicalin were detected by UV absorption at 249 nm and 278 nm, respectively, on a DAD detector coupled to the VP-ODS column. Conditions for mass spectrometer operation were: ionization mode, ESI; nebulizer gas, N₂ (purity 99.999%), flow rate of 1.5 L/min; drying gas, N₂ (purity 99.999%); pressure, 0.1 MPa; interface temperature, 260 °C; heat

block temperature, 230 °C; curved desolvation line (CDL) temperature, 280 °C; detector voltage, 1.5 kV; CDL voltage, 10 V; positive and negative ionization mode, scanning from *m/z* 50 to *m/z* 1000.

2.3. Preparation of standard solutions and samples

Quercetin and baicalin standard solutions (1 mg/ml) were freshly prepared in methanol every week and stored at –20 °C in the dark. Samples of SHL injection were also stored at –20 °C in the dark. All samples for column chromatography were diluted with water to suitable concentrations right before analysis.

2.4. Cell culture and preparation of CMC column

RBL-2H3 cell line was purchased from American type culture collection (ATCC, USA). Cell culture and RBL-2H3/CMC column preparation were performed as previously described [30]. RBL-2H3/cells were cultured in Dulbecco's modified eagle medium (Invitrogen, Grand Island, USA) supplemented with 10% fetal bovine serum, 100 μg/ml penicillin, and 100 U/ml streptomycin. Cells were cultured at 37 °C, 5% CO₂ in a humidified CF EasyFill-2 cell factory (Nunc, Denmark). Cells (7 × 10⁶) were harvested at 80–90% confluency and washed three times with physiological saline (pH 7.4) by centrifugation at 3000 × *g*, 4 °C for 10 min. Cells were resuspended in 50 mM Tris–HCl (pH 7.4), ruptured by 30 min ultrasonication, homogenated for 3 min, and centrifuged at 1000 × *g*, 4 °C for 10 min. The pellet was discarded and the supernatant was centrifuged at 12,000 × *g*, 4 °C for 20 min. The crude membrane precipitation was resuspended in 10 ml physiological saline and centrifuged again at 12,000 × *g*, 4 °C for 20 min. The precipitate was resuspended in 5 ml physiological saline and used in CMC column preparation. To prepare the CMC column, 50 mg silica was activated at 105 °C for 30 min. Cell membrane suspension prepared as above was slowly added to the activated silica under a vacuum at 4 °C with agitation. The mixture was subsequently agitated for 30 min with a magnetic stirrer and let stand for 14 h. Finally, the RBL-2H3/CMC column was produced by packing the stationary phase prepared as above into a column (10 mm × 2.0 mm i.d.) following a wet packing procedure on a column loading machine.

2.5. Validation of RBL-2H3/CMC online LC/MS system

After RBL-2H3/CMC columns were prepared, they were attached to the LC instrument via a ten-port two-position switch valve. The RBL-2H3/CMC column was pre-equilibrated for 1 h before each sample injection. Quercetin, a compound known to induce anaphylactoid reactions [31], was used as a positive control to validate the two-dimensional online system for detection and identification of allergenic agents. The reproducibility of the RBL-2H3/CMC column was assessed by repeated injections of quercetin. For a single RBL-2H3/CMC column, retention times from five repeated quercetin injections (5 μl, 0.1 mg/ml) were recorded and compared. For different RBL-2H3/CMC columns, quercetin (5 μl, 0.1 mg/ml) was injected into each individual column and retention times were recorded and compared. In addition, the durability of the RBL-2H3/CMC column was assessed by repeated quercetin injection (5 μl, 0.1 mg/ml) over a period of 48 h. Quercetin was also used as a positive control to test the suitability and sensitivity of the integrated RBL-2H3/CMC online LC/MS system for allergenic agent isolation and identification. Analysis of SHL injection on the RBL-2H3/CMC online LC/MS system

The potential allergenic components in SHL injection were resolved by the RBL-2H3/CMC column and then analyzed by LC/MS for identification. The identity of resolved allergenic component

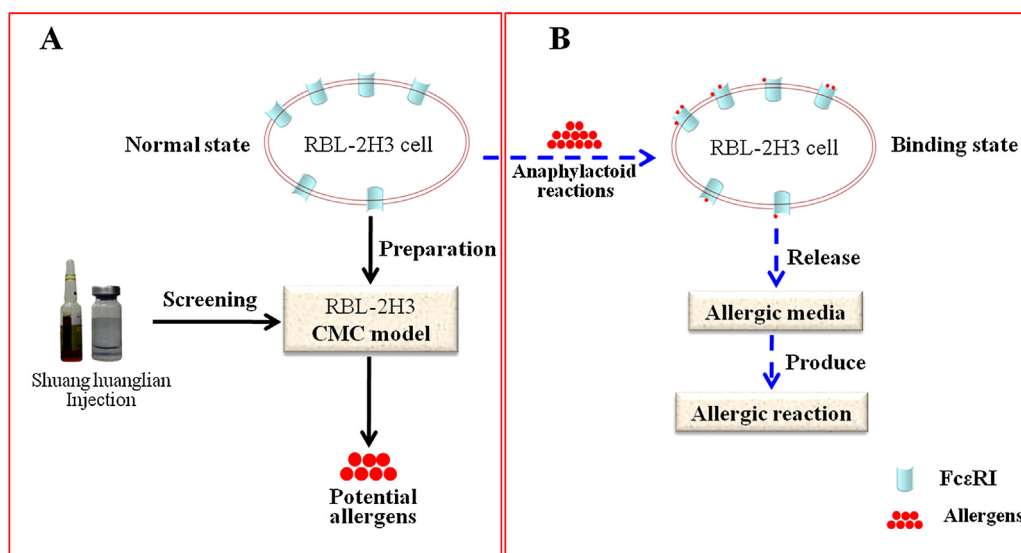


Fig. 1. An overview of screening strategy for allergic components that focused on the IgER. (A) IgER are overexpressed on RBL-2H3 cell membrane which can be used as stationary phase in CMC model. Components of TCM injection retained on RBL-2H3/CMC represented as potential allergen. (B) The role of IgER in the anaphylactoid reactions. IgER cross-linking induced by allergen can lead to the degranulation of mast cells, which may initiate immediate allergic reaction.

was confirmed by side-by-side comparison with standard solution made of pure compound.

2.6. Cell viability assay

RBL-2H3 cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells at exponential growth phase were harvested and plated in 96-well plates at 1×10^5 cells/well. After 24 h incubation at 37 °C, cells were treated with different concentrations of test compound for 48 h. 20 μ L MTT (5 mg/ml) was added to each well and cells were incubated at 37 °C for an additional 4 h. After medium was removed, 150 μ L DMSO was added to each well and optical density at 450 nm was determined on a Bio-rad microplate reader (USA) [32].

2.7. Cell granule staining

RBL-2H3 cell degranulation was assessed by neutral red staining experiment. Briefly, cells were harvested and plated in 24-well plates at 1×10^5 cells/well. After 24 h incubation at 37 °C, cells were treated with different concentrations of test compound for 30 min and subsequently placed on ice for 10 min to stop the reaction. After medium was removed, 400 μ L of 0.5% neutral red was added to each well and cells were incubated at 37 °C for 3 min. The degranulation rate of cells was obtained by counting dyed cells using a cytometer.

2.8. β -Hexosaminidase release assay

Degranulation of RBL-2H3 cells was also measured by the release of β -hexosaminidase, a granule marker. Cells were seeded in 96-well plates at 1×10^5 cells/well and cultured in growth medium for 24 h. The growth medium was then replaced by modified-Tyrod's buffer (119 mM NaCl, 4.74 mM KCl, 2.54 mM CaCl_2 , 1.19 mM KH_2PO_4 , 10 mM HEPES, 5 mM glucose, 0.1% (w/v) BSA, pH 7.3). After 1 h incubation at 37 °C, cells were treated with different concentrations of test compound for 30 min and subsequently placed on ice for 10 min to stop the reaction. The supernatant was collected and incubated with an equal volume of substrate solution (0.2 M citrate, 1 mM

4-methylumbelliferone- β -D-glucosaminide, pH 4.5) for 1 h. 4-Methylumbelliferone was measured by absorbance at 450 nm on a Bio-rad microplate reader (Hercules, USA). To determine the total amount of β -hexosaminidase, the remaining cells were lysed by treatment with assay buffer containing 1% (v/v) Triton X-100 prior to incubation with substrate. Percent β -hexosaminidase release was calculated as the ratio of the absorbance of supernatant to that of cell lysate. Effects of different treatments on β -hexosaminidase release were reported as % of control.

2.9. Statistical analysis

All results are presented as mean $\pm$ SEM. Statistical analysis of differences between groups was performed by two-tailed Student's *t* test. Differences with $p < 0.05$ were considered statistically significant.

3. Results and discussion

3.1. Validation of the RBL-2H3/CMC online HPLC/MS system

The relative standard deviation (RSD) of retention time of quercetin on a single RBL-2H3/CMC column was calculated to be 0.69% over five repeated injections ($n = 5$), indicating excellent reproducibility of column performance. The reproducibility of different RBL-2H3/CMC columns was assessed by injecting quercetin into three RBL-2H3/CMC columns. The relative standard deviation (RSD) of retention time on the three columns was calculated to be 4.65% ($n = 3$), indicating good inter-column reproducibility. We also tested the durability of the RBL-2H3/CMC column over time by making the column work continuously for 48 h. The retention time and peak area of quercetin stayed mostly the same after 48 h continuous work, indicating that the column had sufficient durability for subsequent experiments.

We performed sample analysis on the RBL-2H3/CMC online HPLC/MS system as previously described [33–36]. Briefly, the standard solution of quercetin was used to verify that the online system was capable of screening, analysis, and identification of potential allergenic agents (Fig. 2). As shown in Fig. 2A, the retention time of quercetin was about 2.9 min. The retained fraction R_1 (between the two dotted lines) was enriched onto EC_1 ,

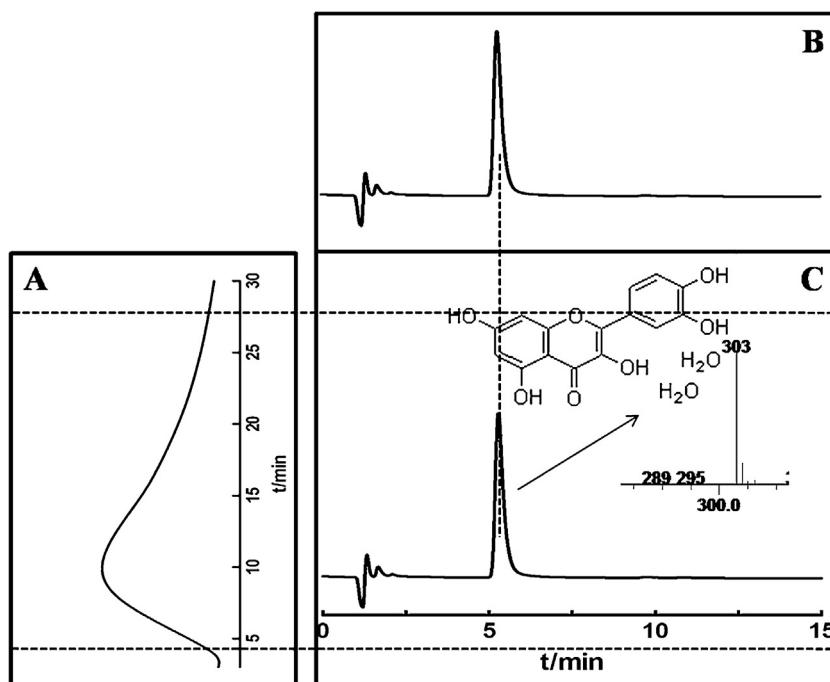


Fig. 2. Results of quercetin analyzed by RBL-2H3 CMC online LC/MS system. (A) Quercetin retained on RBL-2H3 CMC column. (B) The direct LC/MS chromatograms of quercetin standard solution. (C) The retained fraction R_1 (between the two dotted lines in A) was enriched onto EC_1 , and then switched into analytical column for chromatographic separation and MS identification.

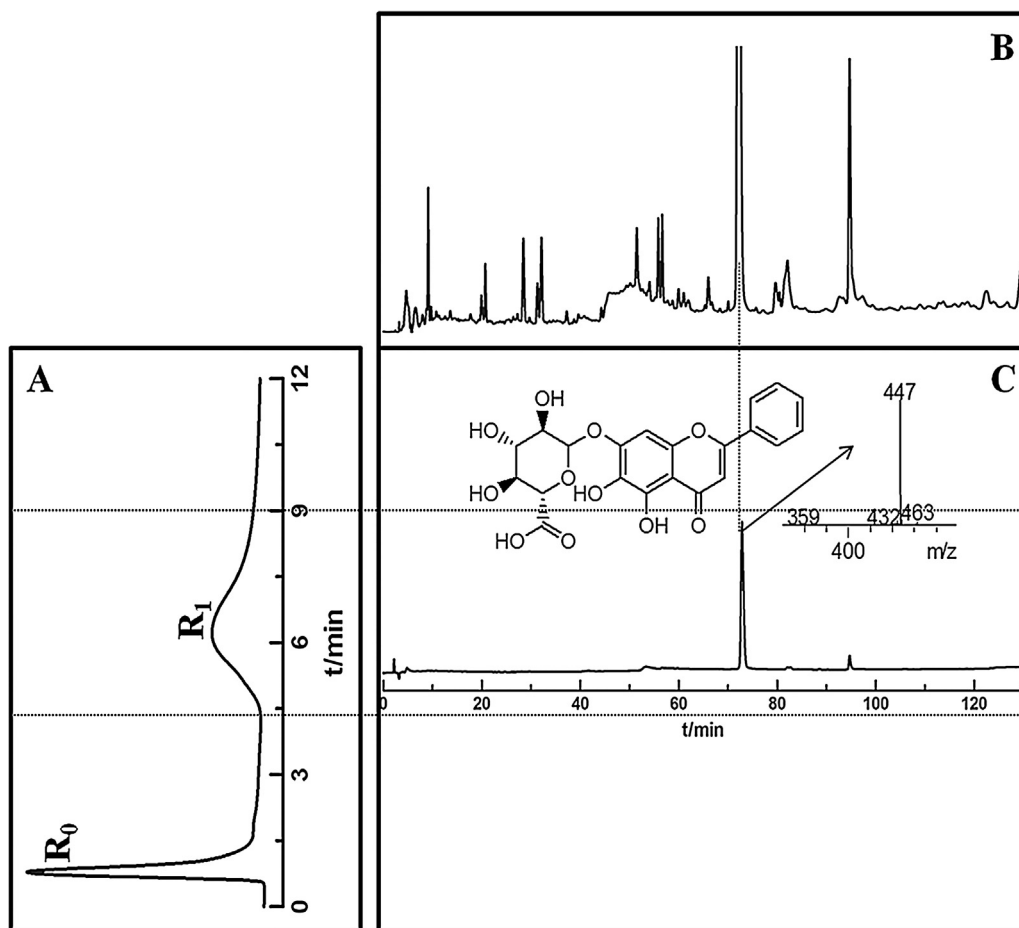


Fig. 3. Results of SHL injection analyzed by RBL-2H3 CMC online LC/MS system. (A) There was one retained fraction R_1 (between two dotted lines) on RBL-2H3 CMC column. (B) The direct LC/MS chromatograms of SHL injection. (C) The LC/MS chromatograms of the retained fraction and identified as baicalin.

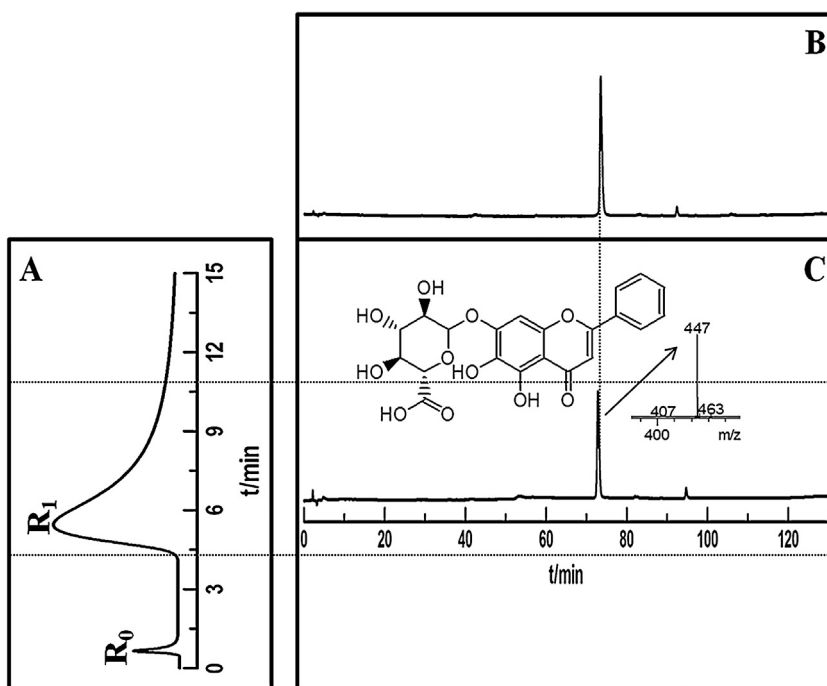


Fig. 4. Results of baicalin standard solution analyzed by RBL-2H3 CMC online LC/MS system. (A) The RBL-2H3 CMC chromatogram of baicalin containing one retained fraction R_1 (between two dotted lines). (B) The direct LC/MS chromatograms of the baicalin standard solution. (C) The LC/MS chromatograms of the retained fraction and identified as baicalin.

and then switched into analytical column for chromatographic separation and MS identification. Data from the second dimensional chromatography and mass spectrometry confirmed that the retained fraction was quercetin (Fig. 2C). The direct LC/MS chromatogram of quercetin is shown in Fig. 2B. Therefore, the RBL-2H3CMConlineLC/MS system is capable of identifying potential allergenic components acting on RBL-2H3 cells.

3.2. Screening allergic components in SHL injection

Next, the RBL-2H3/CMC online LC/MS system was used to screen potential allergenic components in SHL injection. Fig. 3A shows

that one significant retained fraction (R_1) with a retention time of approximately 6 min was separated on the RBL-2H3/CMC column. R_1 was enriched and switched into LC/MS via the switch column valve. LC/MS chromatograms of R_1 and the original SHL injection are shown in Fig. 3C and B, respectively. Data from LC/MS analysis indicated that the retained fraction R_1 was baicalin, a plant flavone previously reported to be a component of SHL injection [13–15]. To verify the identity of R_1 , we tested baicalin standard solution on the online system (Fig. 4). Baicalin standard solution also showed a retention time of about 6 min on the RBL-2H3/CMC column. LC/MS chromatogram of baicalin standard solution (Fig. 4B) and its MS data (Fig. 4C) were identical to those of fraction R_1 from

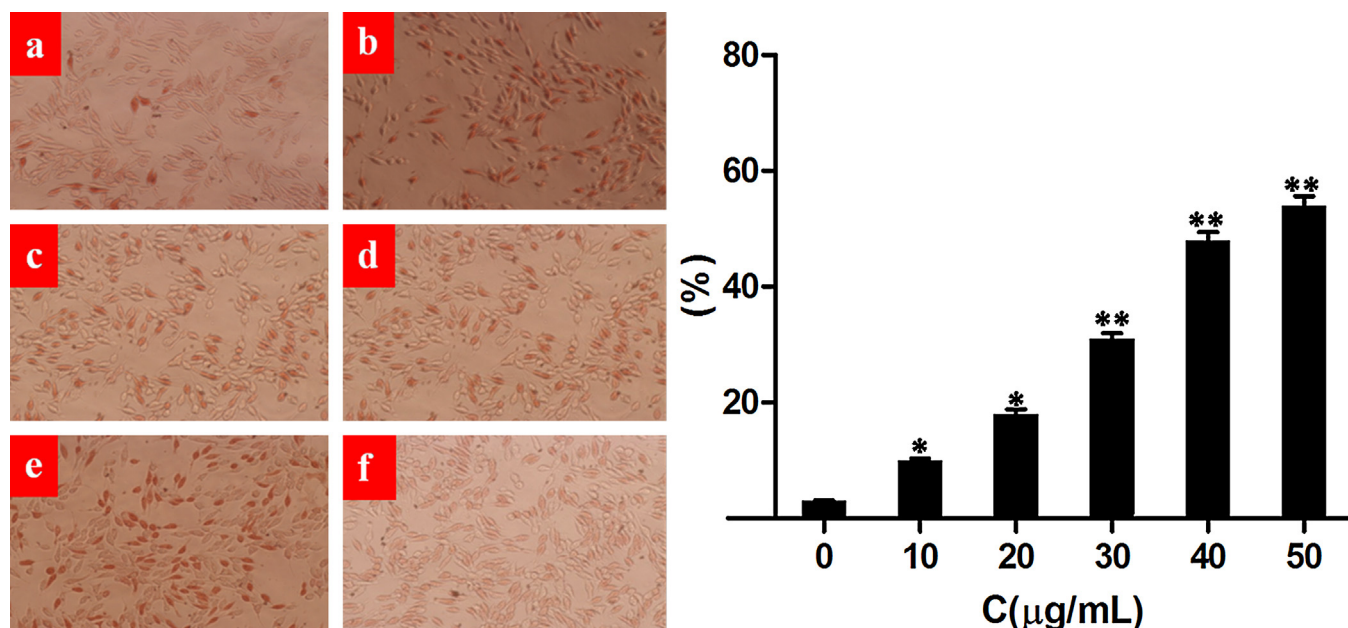


Fig. 5. The effect of baicalin on the cell degranulation of RBL-2H3 cell. Graphs ($\times 200$) of a, b, c, d, e, and f corresponding to 50, 40, 30, 20, 10, and 0 µg/ml baicalin respectively. * $P < 0.05$; ** $P < 0.01$.

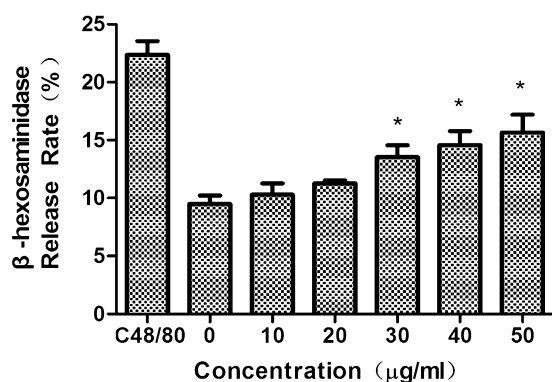


Fig. 6. The effect of baicalin on the release rate of β -hexosaminidase of RBL-2H3 cell. * $P < 0.05$.

SHL injection. Thus baicalin was identified as the potential allergenic component in SHL injection based on its retention by the RBL-2H3/CMC column.

3.3. Cytotoxicity of baicalin on RBL-2H3 Cells

Cytotoxicity of baicalin on RBL-2H3 cells was determined by MTT assay. Baicalin showed dose-dependent cytotoxicity against RBL-2H3 cells as shown in supplementary Fig. 1, which plateaued at 55% inhibition at baicalin concentrations above 50 $\mu\text{g/ml}$. Thus cell degranulation and β -hexosaminidase release were tested at baicalin concentrations from 10 to 50 $\mu\text{g/ml}$ to avoid significant cell death.

Supplementary material related to this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jpba.2013.10.006>.

3.4. Cell granule staining

Mast cell degranulation and mediator release induce allergic reactions. RBL-2H3 cells have similar granular content to that of mast cells, and are thereby commonly employed as a prototypic and convenient model to study mast cell degranulation [37]. In this study, the effect of baicalin on RBL-2H3 degranulation was investigated by neutral red staining. As shown in Fig. 5, baicalin dose-dependently increased the number of red dye-stained cells, indicating that baicalin caused RBL-2H3 cell degranulation in a dose-dependent manner.

3.5. β -Hexosaminidase release

Release of β -hexosaminidase was also measured for corroboration degranulation caused by baicalin stimulation. β -Hexosaminidase is a typical enzyme marker of mast cell degranulation. RBL-2H3 degranulation leads to the release of multiple mediators such as histamine, 5-HT, and β -hexosaminidase in an almost identical manner and constitution as observed in primary mast cells and basophils. We found that baicalin induced β -hexosaminidase release in RBL-2H3 cells in a dose-dependent manner from 10 to 50 $\mu\text{g/ml}$ (Fig. 6), although the effect was less powerful compared with quercetin.

4. Conclusion

In summary, an RBL-2H3/CMC online LC/MS system was established and validated for successful screening and identification of allergenic components in SHL injection. Baicalin was identified as a potential allergenic component and its allergenic activities were confirmed in cell degranulation and β -hexosaminidase release

tests *in vitro*. We believe that the RBL-2H3/CMC-online-LC/MS system developed in this study may be used to screen allergenic components in other TCM injections.

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