



Screening allergic components of Yejuhua injection using LAD2 cell membrane chromatography model online with high performance liquid chromatography-ion trap-time of flight-mass spectrum system

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ABSTRACT

Yejuhua (YJH) injection is a traditional Chinese medicine (TCM) injection and has a widely application in clinical practice. However, adverse drug reactions (ADRs) caused by YJH injection, majorly manifested as allergic reactions, have been reported. Hence, Effective and practical method for allergen screening and identification is needed. In this work, a LAD2/CMC model coupled online with HPLC-IT-TOF-MS system was developed to screen, analyze, and identify the allergenic components of YJH injection. A fraction was retained on the LAD2/CMC column, and identified as linarin (LN). Histamine release assay was performed by the multiple reaction monitoring (MRM) method of UPLC-ESI-MS/MS. Results showed that YJH injection and LN were in accord with their allergic effects by increasing histamine release. In conclusion, the LAD2/CMC-HPLC-IT-TOF-MS system developed in this study could be used to screen allergenic components in complex systems.

1. Introduction

Yejuhua (YJH) injection is one of TCM injections, firstly prepared for clinical use in 1971, and extracted from the single raw medicine *Chrysanthemi Indici Flos* (Yejuhua), which is obtained from the dried capitule anthotaxy of feverfew *Ghrysanthemum indicum* L. YJH injection is widely used in the treatment of upper respiratory tract infection, acute tonsillitis and throat ache [1,2]. However, adverse drug reactions (ADRs) of TCM injections, including anaphylactic shock and fatal anaphylaxis have been reported in a large amount of clinical research and literature articles in Chinese biomedical journals [3–7], and an ADR case of YJH injection was reported in 2016 when used in clinical [8]. Therefore, it is necessary to screen the potential allergens of YJH injection in order to ensure the effectiveness and the safety of the injection, ultimately provide guidelines for the production and quality control of the injection.

To achieve this goal, chromatography can be taken into consideration. High-throughput screening is an important method of identifying target components, such as direct screening based on receptors or enzymes. Cell membrane chromatography (CMC), as a kind of biological affinity chromatography, was firstly developed by He and his

colleagues in 1996 [9]. CMC has been confirmed to be an effective method to screen bioactive components acting on specific receptor from complex systems [10–15]. Besides, the separation and identification of the bioactive components can be accomplished by liquid chromatography-mass spectrum (LC-MS). Thus, a CMC coupled with LC-MS method was chosen in this work.

Mast cells are immune effector cells that participate in the majority of anaphylactic events, and always play important roles in allergic reactions [16,17]. Mast cells can secrete various inflammatory mediators via degranulation process after directly activated by allergens, these released substances include histamine (HA) [18–20], TNF [21], PGs [22], etc., all of which are associated with systemic pseudo-allergic reactions. Several mast cells have been applied in the assessment of anaphylactoid reactions, in which RBL-2H3 [23,24], P815, HMC-1 [25] and Ku812 cell lines are IgE receptor related, while, Stem cell factor (SCF) dependent human mast cell line, Laboratory of allergic diseases 2 (LAD2), established from bone marrow aspirates from a leukemia patient, is related to MRGPRX2 (human G-protein-coupled receptor), which was reported responsible for pseudo-allergic reactions [16]. Hence, the LAD2 cell line was selected as the cell model to distinguish allergic targets from complex systems.

Abbreviations: TCM, traditional Chinese medicine; YJH, Yejuhua; MRM, multiple reaction monitoring; ADRs, adverse drug reactions; CMC, cell membrane chromatography; LC-MS, liquid chromatography-mass spectrum; LAD2, laboratory of allergic diseases 2

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In the present study, LAD2 cells combined with CMC technology, developed previously in our laboratory [26,27], was utilized to establish the LAD2/CMC model. Then the model was employed to screen bioactive compounds of YJH injection, and the screened constituent was enriched, separated, and identified as LN by LCMS-IT-TOF instrument followed by the bioactivity test. We found that LN could induce degranulation of LAD2 cells, resulting in the release of histamine, ultimately causing anaphylactoid reactions. To sum up, the LAD2/CMC-HPLC-IT-TOF-MS system could be used to screen allergenic components in complex systems.

2. Materials and methods

2.1. Chemicals and reagents

LN (HPLC98%, Lot: 20150927) was purchased from Baoji Herbest Bio-Tech Co., Ltd. (Shaanxi, China) and Ciprofloxacin Hydrochloride (CPF, 99.8%, Lot: H0101310) was bought from China Institute of Veterinary Drugs Control (Beijing, China). Compounds including Calcium Ionophore A23187 (A23187), Phorbol 12-myristate 13-acetate (PMA), Atenolol, PD 173074, nimodipine and gefitinib were all obtained from Sigma-Aldrich (USA). YJH injection was supplied by Shanhaidan Pharmaceutical Co., Ltd. (Shaanxi, China). Silica gel (ZEX-II, 5 μ m, 200 Å) was supplied by Qingdao Meigao Chemical Co., Ltd. (Qingdao, China).

HPLC-grade acetonitrile and methanol were purchased from Thermo Fisher Scientific (Pittsburgh, USA). Formic acid for mass spectrometry use was obtained from Sigma-Aldrich (USA). All other reagents involved were of analytical grade or better. Ultra-pure water, produced using a MK-459 Millipore Milli-Q Plus ultra-pure water system, was used throughout the experiment.

2.2. Instrument configuration and chromatographic conditions

The two dimensional analytical system was developed and controlled by a VICIAG 10G-0911V ten-port two-position switching valve (VICI Valco Instrument Co. Inc., Houston, TX, USA). Two Shim-pack VP-ODS pre-columns (10 mm $\times$ 2.0 mm I.D., 5 μ m, Shimadzu Corporation, Kyoto, Japan) were employed as enrichment columns. An LAD2/CMC column (10 mm $\times$ 2.0 mm I.D., 5 μ m, described later in the section of "Preparation of the LAD2/CMC column") was used in the first dimension, and an HPLC analytical column (Diamonsil C18 (2), 250 mm $\times$ 4.6 mm I.D., 5 μ m, Dikma Technologies, Beijing, China) in the second dimension.

For the instrument configuration, the first dimension established in this study consisted of a LC-20AD pump, a SIL-20A auto sampler, a DGU-20A₃ degasser, and a SPD-20A UV/VIS detector (Shimadzu Corporation, Kyoto, Japan). While the second dimension included two LC-20AD pumps, a SIL-20A auto-sampler, a DGU-20A₃ degasser, a SPD-M20A diode array detector, a CBM-20A communication bus module, and the HPLC system was coupled with an Ion Trap-Time of Flight Mass Spectrometer (LCMS-IT-TOF, Shimadzu Corporation, Kyoto, Japan) equipped with an electrospray ionization source operating in positive ion mode. All the modules above were controlled by a compatible computer using Lab Solutions work station (Shimadzu Corporation, Kyoto, Japan).

For the LAD2/CMC system, ultra-pure water (pH 7.4) was used as the mobile phase, and UV detection for LN at 330 nm and for CPF at 272 nm were used, the flow rate was 0.2 mL/min. For the HPLC, a blend of 0.1% (v/v) acetic acid (A) and acetonitrile (B) was used as the mobile phase. Gradient elution was performed as follows: 0–5 min, 8% B; 5–15 min, increasing linearly to 12% B; 15–25 min, 12% B; 25–60 min, progressing linearly to 35% B and then lasting for 5 min. The injection volume was 20 μ L, trapped over a period of 65 min when using a flow rate of 1.0 mL/min. The temperature of both the LAD2/CMC column and the HPLC column was maintained at 37 °C in a CTO-

20A column oven. The optimal MS parameters were as follows: Nebulizer gas (N₂, purity > 99.999%) flow rate at 3 L/min and drying gas (N₂, purity > 99.999%) pressure at 109 kPa; Interface, ESI source; Curve desolvation line (CDL) temperature at 200 °C, heat block temperature at 200 °C; Interface voltage, 4.5 kV, detector voltage, 1.57 kV; CID gas (Ar, purity > 99.999%), CID energy 50%; Ion accumulation, 30.0 ms, positive ionization scan mode, manual precursor ion selection, and scanning from m/z 100 to 1000. A three-port flow divider valve was employed post column to split the fluid into 0.3 mL/min flowing into MS detector and 0.7 mL/min into drain.

2.3. Preparation of standard solutions and sample solutions

For chromatography analysis use, Standard stock solutions of LN, CPF, atenolol, PD 173074, nimodipine and gefitinib (1.0 mg/mL each) were prepared in methanol weekly and stored at –20 °C in the dark. For cell assay use, LN, A23187 and PMA were all dissolved in DMSO to obtain individual stock solutions at concentrations of 400 mM, 10 mM and 1 mM and stored at –20 °C in the dark.

The stock solutions of HA and d_4 -histamine (d_4 -HA, used as internal standard) were manually prepared in 50/50 (v/v) acetonitrile/water (50 μ g/mL each), a working solution of d_4 -HA was prepared in acetonitrile freshly (with its working concentration in 5 ng/mL).

YJH injection was filtered through a 0.22 μ m filter and stored at –20 °C in the dark.

2.4. Cell culture

The LAD2 human mast cells were kindly provided by A. Kirshenbaum and D. Metcalfe (NIH, USA). LAD2 cells were cultured in StemPro-34 SFM medium (Invitrogen) supplemented with 2 mM L-glutamine, 100 U/mL streptomycin, 100 μ g/mL penicillin, and 100 ng/mL recombinant human SCF (Peprotech), grown on a flask, and incubated at 37 °C in the 5% CO₂ atmosphere. Cells from exponentially growing cultures were used in all experiments.

2.5. Preparation of the LAD2/CMC column

Plenty of LAD2 cells (1×10^7 cells/mL) were collected when the cells were in exponential phase. The harvested LAD2 cells were first washed three times with physiological saline (pH 7.4) by centrifugation at 3000g, 4 °C for 10 min, and then ruptured by sonic disruption (JY92-II ultrasonic cell disruptor) in an ice-bath for 30 min. After that, the suspension were homogenized for 3 min, and then separated by centrifugation at 1000g, 4 °C for 10 min. Only the supernatant was collected and centrifuged at 12,000g, 4 °C for 20 min to obtain the precipitation which was then suspended in 10 mL of physiological saline, and followed by centrifugation at 12,000g, 4 °C. 5 mL of physiological saline was then added to re-suspend the LAD2 cell membrane precipitate. The cell membrane suspension was slowly added to the silica (50 mg, activated at 105 °C for 30 min) under vacuum with continuous vortex in an ice-bath. The mixture was then stirred for 30 min using a magnetic stirrer and left to stand overnight at 4 °C to produce the LAD2 cell membrane stationary phase (CMSP) which was finally packed into a CMC column (10 mm $\times$ 2.0 mm) on a column-loading machine following a wet packing method.

2.6. Method validation

In this study, we developed a method of two-dimensional LAD2/CMC coupled online with HPLC-IT-TOF-MS via a ten-port two-position switching valve and two C18 enrichment columns in order to screen allergic components of YJH injection. LAD2/CMC was used to screen and retain the allergenic components which were then separated and identified by HPLC-IT-TOF-MS system. Before screening procedure, various drugs acting on specific receptors were used to verify the

selectivity of the LAD2/CMC column, and CPF was selected as the positive control drug to investigate the suitability of the LAD2/CMC-online-HPLC-IT-TOF-MS system for this study. CPF standard solution was first injected into the LAD2/CMC column, then got enriched on the pre-column, and finally switched to the HPLC-IT-TOF-MS where it was further identified. Positive and negative drugs were used to investigate the selectivity of the LAD2/CMC column.

2.7. Practical application of the coupled system

The developed method was then used to screen active components of YJH injection after being investigated. For further identification, the corresponding standard compound of the retained constituent identified by the online system was also analyzed by the online-coupled system.

2.8. Histamine release assay

LAD2 cells were cultured in the conditions described before in Section 2.4. Cells from exponentially growing cultures were planted to a 96-well plate at 3×10^4 cells per well, cultured in growth medium, and incubated at 37 °C in a 5% CO₂ atmosphere overnight. Drugs to be tested were all diluted from their stock solutions using TM buffer to obtain positive control drugs (A23187 with a final concentration of 5 µM mixed with PMA with a final concentration of 50 nM) and LN with series concentrations of 5, 20, 50, 100, 200 µM. YJH injection was diluted according to the same procedure to get drugs with different dilution folds. All the prepared compounds and samples were added (100 µL per well) to stimulate cells after the plate was centrifuged and the medium was discarded. Then, the plate was incubated at 37 °C in the 5% CO₂ atmosphere for 30 min, immediately following the incubation the culture supernatants (50 µL per well) were collected into 1.5 mL tubes and left to stay on ice for 10 min to stop the reaction. Specimens were precipitated with acetonitrile (100 µL per tube, with d₄-HA dissolved in it) and centrifuged (12,000g, 10 min, 4 °C), the supernatant of each sample was collected for Mass Spectrometry analysis. It is worth mentioning that all the procedures above were performed on ice bath. Histamine release assay was performed by a UPLC-ESI-MS/MS method with conditions as follows: a HILIC column (Venusil HILIC, 2.1 mm × 150 mm, 3 µm, Agela Technologies, Tianjin, China) was employed, and an isocratic elution with a blend of acetonitrile and water added with 0.1% formic acid and 20 mM ammonium formate (80: 20, v/v) was adopted at a flow rate of 0.3 mL/min.

2.9. Statistical analysis

All values were expressed as mean ± SEM (standard error of the mean). The SPSS 18.0 software was used to analyze statistical data and a two-tailed non-paired Student's *t*-test was performed to determine the differences between two groups with *P*-values less than 0.05 considered significant.

3. Results and discussion

3.1. Validation of the LAD2/CMC online with HPLC-IT-TOF-MS method

CPF, atenolol, PD 173074, nimodipine and gefitinib were first analyzed by the first-dimensional column and the chromatograms obtained using LAD2/CMC are shown in Fig. 1. The four negative drugs act on specific receptors: atenolol acts on the β-receptor, PD 173074 is a fibroblast growth factor receptor 3 (FGFR3) inhibitor, nimodipine acts on the calcium channel and gefitinib on the EGF receptor. It was reported that CPF could act on hMRGPRX2. As we can see in Fig. 1, the chromatogram of CPF included non-retained and retained fractions, while other four compounds just showed non-

retained fractions consisting of standard compounds and solvent constituent, suggesting that only CPF was retained on the column, which demonstrated that CPF had better retention characteristic, and indicated that the LAD2/CMC model could screen specific compounds selectively.

For its retaining characteristic on LAD2/CMC column, CPF was used as a control drug acting with the LAD2 cells in the research of anaphylactic reactions. After the selectivity assay, CPF was first analyzed using the whole coupled system, and the results are shown in Fig. 2. Fig. 2a shows the chromatogram of the retained fraction (R₁, with the retention time of about 55 min) on the LAD2/CMC column. After the target component (between the two dotted lines) was recognized and enriched, it was then switched to HPLC-IT-TOF-MS for further analysis. The chromatogram of the retained fraction (R₁) in Fig. 2c performs the same as that obtained from a direct injection with CPF in Fig. 2b. Based on the results shown in Fig. 2, the LAD2/CMC coupled with HPLC-IT-TOF-MS method can effectively determine components that act on the cell membrane.

In recent years, the increasingly popular chromatography/mass spectrometry, chip technology, genomics technology, computer virtual screening, and other techniques have been widely used in the screening of anaphylactoid components. In fact, these screening methods generally require a single (pure) compound for analysis, and are difficult to be applied in identifying the target components from a complex system without isolation and purification processes. In our study, the LAD2/CMC coupled with HPLC-IT-TOF-MS method can effectively determine components from complex system directly.

3.2. Practical application of the coupled system

The established method was applied to screen potential allergic components of YJH injection (Fig. 3). As is shown in Fig. 3a, one main retained fraction (R₁, with its *t_R* at about 5 min) was got by the LAD2/CMC model, eluted after a high intensity non-retained fraction (R₀). The retained fraction, R₁, was enriched and switched to the HPLC-IT-TOF-MS system (shown in Fig. 3c), being identified as LN, with its precursor ion at *m/z* 593.2226. The chromatogram of YJH injection with direct injection (Fig. 3b) showed the same characteristics, both in retention behavior and their precursor ions. For further identification of the screened component, the standard compound of LN was also analyzed through the whole system with a similar “screen-enrich-switch-identify” mode (Fig. 4), results showed a similar retention time of LN retained on the LAD2/CMC column, and displayed the same retention behavior and MS precursor on the second dimension. Hence, we concluded that the screened component of YJH injection was indeed LN.

3.3. Histamine release assay

Human LAD2 cells were used as an allergy model to assess allergenic effect induced by drugs. In addition, mast cells usually undergo a degranulation process, resulting in the release of many different types of biological substances, including HA, that mediate manifestations. While HA is one of the best characterized mediators for allergic responses, histamine release assay was performed to assess degranulation of LAD2 cells induced by LN and YJH injection in this work. In the histamine release assay, a selected reaction monitoring (SRM) mode was used to achieve quantification of HA in complex mixtures, in the SRM experiment for HA, a predefined precursor ion (*m/z* 112.00) and three of its fragments were selected (*m/z* 95.05, 68.05, 41.05) by the two mass filters of a triple quadrupole instrument and monitored over time for precise quantification, and for the internal standard d₄-HA, the transitions were *m/z* 116.00 > 99.10, *m/z* 116.00 > 72.00, and *m/z* 116.00 > 44.00, the cone voltage was 4.5 kV. Collision energy (CE) for HA was −15.0, and for d₄-HA, the CE value was −19.0. In this work, after stimulating LAD2 cells by LN with concentrations from 5 to 200 µM and YJH injection with different

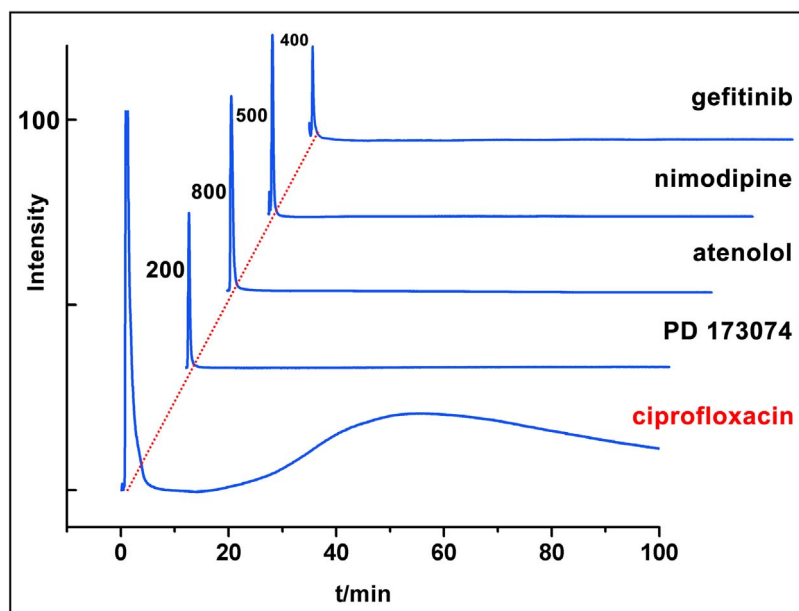
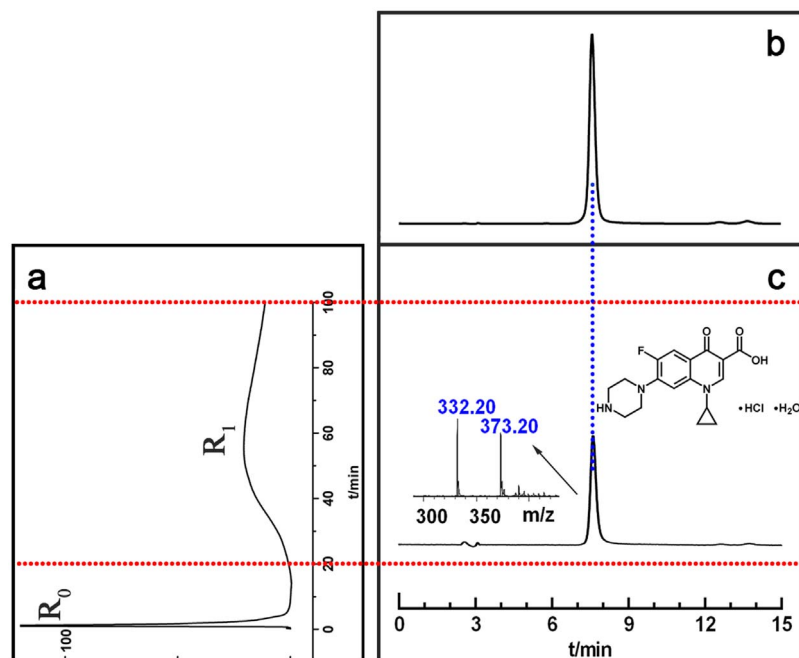


Fig. 1. The selectivity of the LAD2/CMC column.

Fig. 2. Chromatograms of CPF standard solution analyzed by LAD2/CMC coupled with HPLC-IT-TOF-MS method. a. Chromatogram of standard solution of CPF retained on the LAD2/CMC column. b. Direct HPLC-IT-TOF-MS chromatogram of CPF. c. HPLC-IT-TOF-MS chromatogram of retained fraction (R_1).

dilute folds from the original injection, the amount of released histamine was detected. As shown in Fig. 5a, the amount of HA released from LAD2 cells stimulated with LN was higher than 100 ng/mL, showing a marked difference (with $P < 0.01$) compared with control group (about 30 ng/mL) and positive drug group (around 250 ng/mL), the results in Fig. 5b exhibited to be similar. Therefore, results from Fig. 5 demonstrated that LN could increase the release of HA in LAD2 cells, as shown in Fig. 5a. Accordingly, all the YJH injection solutions could induce the release of HA in LAD2 cells, as shown in Fig. 5b. From the results in Fig. 5, we can safely draw the conclusion that LN in YJH injection could indeed induce degranulation in LAD2 cells.

4. Conclusions

In summary, an online LAD2/CMC-HPLC-IT-TOF-MS system was developed to selectively screen active components binding with LAD2 cells in this study, then, the method was applied to screen potential allergic constituents in YJH injection, and a retained fraction was analyzed and identified as LN. Histamine release assay was carried out to verify the activity of LN *in vitro* and results showed that LN could induce degranulation in activated mast cells. Thus, this coupled system has the potential to serve as a useful tool for screening allergenic components of natural medicinal herbs.

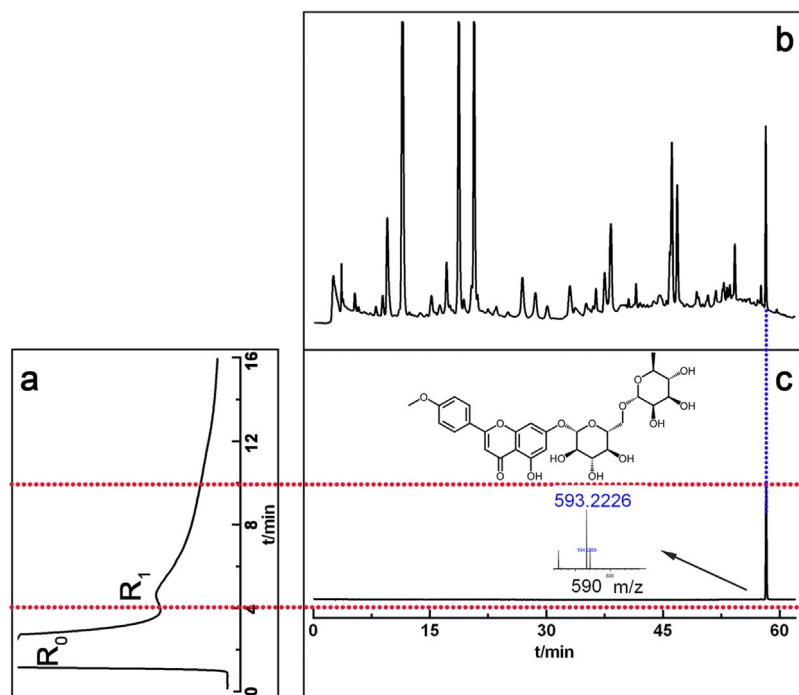


Fig. 3. Chromatograms of YJH injection analyzed by LAD2/CMC coupled with HPLC-IT-TOF-MS method. a. Chromatogram of YJH injection retained on the LAD2/CMC column. b. Direct HPLC-IT-TOF-MS chromatogram of YJH injection. c. HPLC-IT-TOF-MS chromatogram of the retained fraction (R₁).

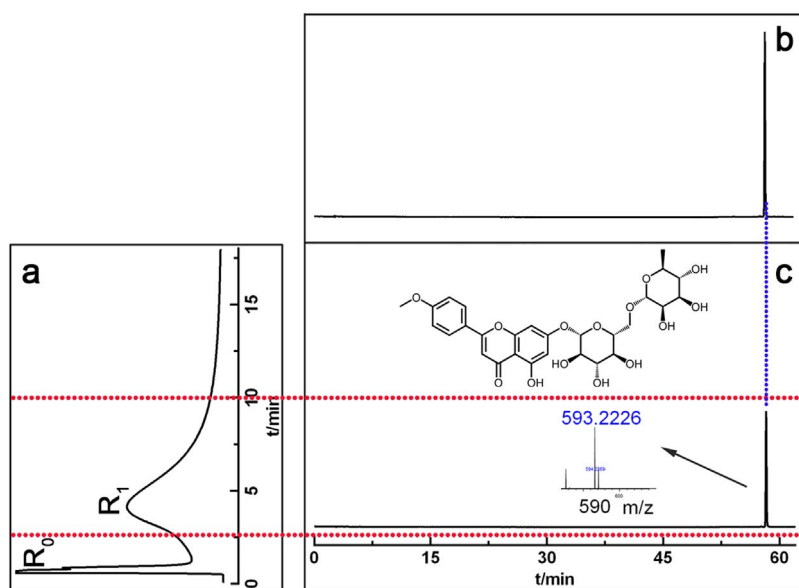


Fig. 4. Chromatograms of the standard solution of LN analyzed by LAD2/CMC coupled with HPLC-IT-TOF-MS system. a. Chromatogram of standard solution of LN retained on the LAD2/CMC column. b. Direct HPLC-IT-TOF-MS chromatogram of the standard solution. c. HPLC-IT-TOF-MS chromatogram of the retained fraction (R₁).

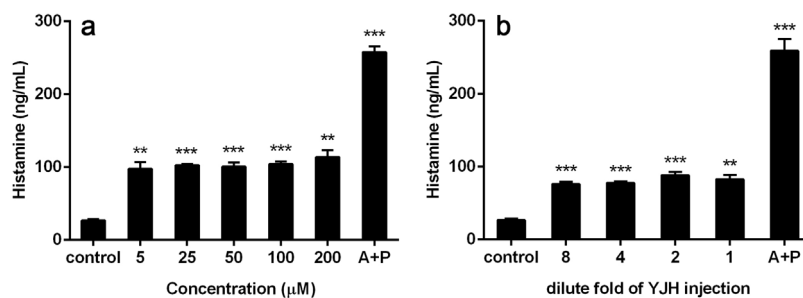


Fig. 5. The effect of LN and YJH injection on the release of histamine of LAD2 cells. a. The histamine release assay of LAD2 cells induced by LN. b. The histamine release assay of LAD2 cells stimulated by YJH injection. * $P < 0.05$, ** $P < 0.01$.

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