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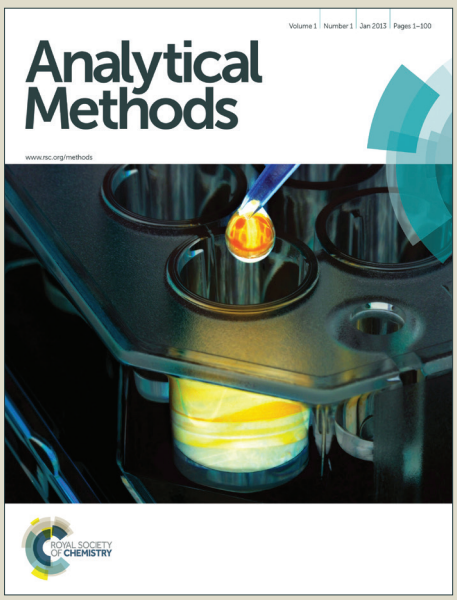

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A sensitivity HPLC-ECD method for detecting serotonin released by RBL-2H3 cells stimulated by potential allergens

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Abstract

A high sensitive high performance liquid chromatography-electrochemical detection (HPLC-ECD) method being used to detect released serotonin in real time from rat basophilic leukemia 2H3 (RBL-2H3) cells which can be stimulated by potential allergens, was established to evaluate the sensitization of potential allergens. The chromatographic separation was carried out on a Chromolith ® Speed ROD RP-18e column (50 mm × 4.6 mm I.D., 2 μm). The linearity of serotonin in samples was good, with correlation coefficients greater than 0.9986 within the corresponding concentration range. The relative standard deviation (RSD) percentages were in the range of 0.72%–2.96% for inter-day precision and in the range of 2.02%–3.48% for intra-day precision. The relative errors (RE) were within ±3.21%.The recovery of serotonin was in the range of 100.98%–101.56%, and the RSDs of recovery were less than 2.28%.The method allows for simple sample preparation, short analysis time, and high sensitivity, specificity, and reliability. This method was successfully used to detect serotonin released by RBL-2H3 cells stimulated by different concentrations of schisandrin A and harpagoside. The results demonstrate that schisandrin A and harpagoside can trigger RBL-2H3 cells to release serotonin in a dose-dependent manner. The study contributes to the further use of evaluation of potential allergens.

Keywords

high performance liquid chromatography-electrochemical detection, rat basophilic leukemia 2H3 cells, serotonin, potential allergens, schisandrin A, harpagoside

1. Introduction

Acute allergic reactions including type I anaphylaxis and anaphylactoid reactions, are common clinical adverse drug reactions [1,2]. When allergic reactions occur, various mediators such as histamine, serotonin, and β-hexosaminidase are released and can cause a series of systemic reactions, such as smooth muscle contraction, vasodilation, vascular permeability increase, anaphylactic shock, blood or serum sickness reaction, and even be fatal [3-5].In order to control allergic reactions, screening and evaluating potential allergens is an important task.

The rat basophilic leukemia (RBL) cell line was developed in 1973 by inducing leukemia in rats fed with the chemical carcinogen, β-chlorethylamine [6]. In view of their similar granular content to mast cells, RBL-2H3 cells are commonly employed as a prototypical and convenient model to study allergies [7]. Allergic mediators such as histamine, serotonin, and β-hexosaminidase are released by RBL-2H3 cells upon stimulation by potential allergens [8,9]. Usually, the amount of histamine or β-hexosaminidase released by RBL-2H3 cells can be used to evaluate the sensitization of potential allergens. Due to the short half-life of histamine in the biological sample, the sample had to be derived before HPLC detection, and accurate quantitative detection of histamine was difficult [10]. Because the serotonin released by RBL-2H3 cells was associated with histamine [11], serotonin and β-hexosaminidase can be used as indicators to evaluate sensitization.

Therefore, highly sensitive analytical method for detection of released serotonin

from RBL-2H3 cells, which are stimulated by potential allergens was urgently needed to evaluate potential allergens. There are many methods that have been reported to detect serotonin, such as HPLC-UV detection, HPLC-fluorescence detection, and enzyme-linked immune sorbent assay (ELISA) [12-14]. However, HPLC-UV detection and HPLC-fluorescence detection need derivatization, the process is complex [15, 16]. The ELISA method takes too long to perform and is not sensitive enough [17]. And there was a large amount of volatile salt in the sample after RBL-2H3 cells stimulated by allergens, it was not suitable for detection by mass spectrometry. In this study, we built a sensitive HPLC-ECD method for detection of released serotonin from RBL-2H3 cells stimulated by potential allergens. This method provides for simple sample preparation, short analysis time, and high sensitivity, specificity, and reliability compared with other technologies. The strategy of evaluation of the potential allergens by determination of released serotonin from RBL-2H3 cells by the HPLC-ECD method was as below. Compound 48/80, which was a positive drug to study allergies, was used to stimulate RBL-2H3 cells in the method development process [18]. A β-hexosaminidase release assay was performed by a biomedical method to verify the results. Schisandrin A and harpagoside, from traditional Chinese medicine, were previously reported as potential allergens [19,20]. So, this method was used to detect the released serotonin from RBL-2H3 cells stimulated with different concentrations of schisandrin A or harpagoside, and expect to evaluate the sensitization of the two potential allergens.

2. Materials and methods

2.1 Materials and reagents

Serotonin (>98, % purity, Lot # 10149287) was obtained from Tianjin Alfa Aesar Chemical Co., LTD. (Tianjin, China). Compound 48/80 was obtained from Sigma-Aldrich Co. LLC. Schisandrin A (>98, % purity, Lot # MUST-11121504) and harpagoside (>98, % purity, Lot # MUST-11071801) were bought from Chengdu Must Bio-tech. Co., LTD. (Chengdu, China). Sodium dihydrogen phosphate and EDTA-2Na were bought from Xi'an Chemical Reagent Factory (Xi'an, China). Fetal bovine serum and pancreatin were bought from Invitrogen Corporation (Grand Island, USA).HPLC grade methanol and acetonitrile were obtained from SK Chemicals Co., Inc. (Ulsan, Korea). All aqueous solutions were prepared using ultrapure water which is produced by a MK-459 Millipore Milli-Q Plus ultra-pure water system.

2.2 Instruments and chromatographic conditions

The HPLC system included a DGU-20A_3 degasser, two LC-20AD pumps, a SIL-20A auto sampler, a CTO-20AC column oven, an LC-ECD-6A electrochemical detector, and a Lab-solution workstation (Shimadzu Corporation, Kyoto, Japan). A Chromolith® SpeedROD RP-18e column (50 mm×4.6 mm I.D., 2 μm) was used. Isocratic elution with a flow of 1.0 mL/min was performed with two different mobile phases. Mobile phase A was bufer composed of 0.1 M sodium dihydrogen phosphate and 0.5 mM EDTA-2Na, pH 3.5 (sulfuric acid). Mobile phase B was methanol. The mobile phase ratio was 95% mobile phase A, and 5% mobile phase B. The

temperature of the column was maintained at 37 °C. The detection voltage of the electrochemical detector was set to 0.6V.

2.3 Standard and sample preparation

Compound 48/80, serotonin, schisandrin A and harpagoside standard solutions were separately prepared in methanol (1 mg/mL). The solutions were freshly prepared each week and stored at -20 °C in darkness. When these standard solutions were used, they were diluted to suitable concentrations using freshly prepared mobile phase.

Cells from exponentially growing cultures were used in the sample preparation for all experiments. RBL-2H3 cells were grown on 96-well plates (1×10^5 cells/well). Cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2.The growth medium was replaced by modified-Tyrode'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, and 0.1% (w/v) BSA, pH 7.4).When cells reached approximately 80% confluence, they were treated with Compound 48/80, schisandrin A, and harpagoside at different concentrations. After 45 min of incubation at 37 °C, the 96-well plates were placed on an ice bath for 10 min to stop the reaction. Then, the supernatant was transferred and filtered using a Millipore filter (0.45 μm), and the sample work solution was stored at -20°C in the dark until used for chromatography.

2.4 Optimization of the chromatographic conditions

In order to shorten the analysis time, and get a good peak shape the concentration of buffer, the pH, and the ratio of buffer with methanol in the mobile phase were

optimized. The voltage of the ECD detector and sample volume were also optimized to make the method more sensitive.

2.5 Method Validation

The selectivity, linearity, sensitivity, precision, accuracy, recovery, and stability of the HPLC-ECD method were validated according to the bioanalytical method validation guide published by the Food and Drug Administration (FDA) [21].

2.5.1 Selectivity

The selectivity was evaluated by analyzing blank samples (culture medium), blank samples with added serotonin, and actual samples, respectively.

2.5.2 Linearity

The calibration standards of serotonin were in the concentration range of 10-1000 ng mL^{-1}. The concentrations 10 ng mL^{-1}, 50 ng mL^{-1}, 100 ng mL^{-1}, 200 ng mL^{-1}, 400 ng mL^{-1}, 800 ng mL^{-1}, 1000 ng mL^{-1} were injected into the HPLC system, and the peak areas were recorded. Calibration curves were established by plotting the peak area versus serotonin concentration (X-axis).

2.5.3 Sensitivity

The sensitivity was shown by the lower limit of quantification (LLOQ). The LLOQ was defined as the lowest concentration of serotonin on the calibration curve with a relative standard deviation (RSD) lower than 20% and deviations from the nominal concentration within ± 20% by five replicate analyses.

2.5.4 Precision and accuracy

The precision and accuracy were investigated (at low, middle and high concentrations) for samples in five replicates, which were prepared and analyzed on three consecutive days. RSD was used to evaluate the intra- and inter-day precision. To assess the accuracy, the relative error (RE) was calculated according to the formula: RE% = [(assayed value – nominal value) / nominal value] × 100%.

2.5.5 Recovery

The recovery of serotonin was measured at three different concentration levels (n = 5 for each concentration).The recovery was calculated according to the formula: Recovery = [(analyzed value – nominal value) / added value] × 100%.

2.5.6 Stability

The stability was investigated by analyzing five replicates of the samples at three QC levels under different conditions, including 24 h storage at ambient temperature, three freeze/thaw cycles, and storage at -75 °C for 30 d. The samples were considered stabile the average percentage concentration deviation was within 15% of the actual value.

2.6 Time-effect relationship of released serotonin from RBL-2H3 cells

The time-effect relationship of released serotonin from RBL-2H3 cells was investigated by injecting samples into the HPLC system after RBL-2H3 cells were treated with compound 48/80 treated (5–60 min, 5 min intervals, except the 25, 35, and 55 min time points. The concentration of serotonin at each of these time points was calculated. Then, the time-effect relationship of released serotonin from

RBL-2H3 cells was established by plotting the concentrations of the serotonin versus time (X-axis).

2.7 β-hexosaminidase release assay

Another mediator, β-hexosaminidase, was detected to verify the time-effect relationship of an allergic mediator released by RBL-2H3 cells. β-Hexosaminidase is typically an enzyme marker of mast cell degranulation. RBL-2H3 degranulates upon treatment with multiple mediators, including histamine, serotonin, and β-hexosaminidase, in an almost identical manner, and the degranulation mechanism is the same as observed in primary mast cells and basophils [22]. In this study, RBL-2H3 cells were grown in 96-well plates (1×10^5 cells/well). The growth medium was replaced by modified- Tyrode's buffer. After 1 h incubation at 37 °C, cells were treated with compound 48/80 for 30 min, and then placed on an ice bath for 10 min to stop the reaction. At the end of the experiment, the supernatant was incubated with an equal volume of substrate solution (0.2 M citrate, 1 mM 4-methylumberlliferyl-Nacetyl-β-D-glucosaminide, pH 4.5). Then 100 μL of 0.2 M Tris-HCl buffer, pH 11 was used to rupture cells for 5 min. The absorbance of 4-methylumbelliferone was measured with a Microplate Reader (Bio-rad, Hercules, USA) at 450 nm. To determine the total amount of β-hexosaminidase released, the remaining cells were lysed by treatment with assay buffer containing 1% (v/v) Triton X-100 prior to incubation with substrate using the same procedure as for determination of activity in the supernatant. The amount of β-hexosaminidase release

was calculated by dividing the absorbance of the supernatant by that of the cell lyaste. The effects of different treatments on β-hexosaminidase are reported as percentage of control. The time-effect relationship of released β-hexosaminidase from RBL-2H3 cells was investigated to verify the results of section 2.6.

2.8 Practical application of the HPLC-ECD method

Schisandrin A and harpagoside are two potential allergenic components screened from traditional Chinese medicine in previous study [19,20]. In this study, the HPLC-ECD method was used to verify the sensitization of the two potential allergenic components.RBL-2H3 cells were grown in 96-well plates (1×10^5 cells/well). The growth medium was replaced by modified- Tyrode's buffer. After 1 h of incubation at 37 °C, cells were treated with different concentrations of schisandrin A and harpagoside for 30 min and placed on ice bath 10 min to stop reaction. Then, the culture supernatant was collected and analyzed by HPLC-ECD as in section 2.2. In order to verify the results identified by the HPLC-ECD method, a β-hexosaminidase release assay was performed to verify the sensitization of the two potential allergens.

3. Results and discussion

3.1 Optimization of the chromatographic conditions

In this study, a Chromolith® Speed ROD RP-18e column (50 mm × 4.6 mm I.D., 2 μm) was used to analyze serotonin released by RBL-2H3 cells. Satisfactory chromatographic behavior of serotonin was determined. Isocratic elution with a flow

of 1.0 mL min^{-1} was performed by using two different mobile phases. Mobile phase A was bufer composed of 0.1 M sodium dihydrogen phosphate and 0.5mM EDTA-2Na, pH 3.5 (sulfuric acid). Mobile phase B was methanol. The mobile phase ratio was 95% mobile phase A, and 5% mobile phase B. The retention time of serotonin was less than 6 min. The analysis time was relatively shorter than those in previous methods [23, 24]. The relationship between the voltage of the ECD detector, sample volume, and response intensity is shown in Fig.1. . The intensity was the highest when the voltage of ECD detector was 0.6 mV. In addition, when the sample volume was 10 μL, the response intensity was less volatile near 0.6 mV. Therefore, the voltage of ECD detector was fixed at 0.6 mV, and sample volume was 10 μL in the following study.

3.2 Method Validation

3.2.1 Selectivity

No endogenous interference was observed at the retention time of serotonin (5.6 min). Typical chromatograms of blank samples (culture medium), blank samples with added serotonin, and actual samples, are shown in Fig.2.

3.2.2 Linearity

The typical calibration curves, correlation coefficients and linear ranges of serotonin were as follow. A=344029C-1634, A: peak area of serotonin, C: concentration of serotonin. The regression coefficient (r) was 0.9986. The linear range was from 10 ng mL^{-1} to 1000 ng mL^{-1}.

3.2.3 Sensitivity

The lower limit of quantification of serotonin was always less than 10 ng/mL with good precision and accuracy, which indicated that the method was sufficiently sensitive. And this was more sensitivity than HPLC-UV method 32.5 ng/mL [25] and HPLC-fluorimetric detection 62 ng/mL [26].

3.2.4 Precision and accuracy

The results of accuracy and precision for intra-day and inter-day QC samples at three concentrations (150, 300, 700 ng mL^{-1}) were summarized in Table 1. The RSD was in the range of 0.72%–2.96% for inter-day precision, and in the range of 2.02%–3.48% for intra-day precision. The REs were within ±3.21%.These results showed that the HPLC-ECD method was accurate and could reproducibly determine serotonin concentration.

3.2.5 Recovery

Recovery of the serotonin used in the HPLC-ECD method is shown in Table 2. At three concentration levels of serotonin (150, 300, 700 ng mL^{-1}), the recovery of serotonin was in the range of 100.98%–101.56%. And the RSD of recovery of serotonin was less than 2.28%.These results indicated that the recovery of the HPLC-ECD method for determining the serotonin concentration was acceptable.

3.2.6 Stability

The stability was investigated by analyzing five replicates of the samples at three QC levels under three different conditions, which the samples may experience,

including24 h storage at ambient temperature, three freeze/thaw cycles, and storage at -75°C for 30 d. The results shown in Table 3 demonstrated that the serotonin was stable after 24 h storage at ambient temperature (RSD% was less than 13.87%, RE% was within ±6.1), three freeze/thaw cycles (RSD less than 11.23%, RE within ± 6.1%), and storage at -75°C for 30 days (RSD less than 10.28%, RE within ±6.2%). These results indicated that the stability of the HPLC-ECD method for determining serotonin concentration was acceptable.

3.3 Time-effect relationship of released serotonin from RBL-2H3 cells

The time-effect relationship of released serotonin from RBL-2H3 cells, which had been stimulated by potential allergens, is shown in Fig.3A. Between 5 min and 15 min, the serotonin released by RBL-2H3 cells was stable, but the amount of released serotonin was low. Between 20 min and 30 min, the serotonin released by RBL-2H3 cells increased as treatment time increased. Between 40 min and 60 min, the serotonin released by RBL-2H3 cells was stable, and the amount of serotonin released was high. In order to increase the sensitivity and reduce the system error, RBL-2H3 cells were stimulated for 45 min to release serotonin. This result was verified with a β-hexosaminidase release assay, and the results are shown in Fig.3B. Between 40 min and 60 min, β-hexosaminidase release leveled off. So, the time-effect relationship of released β-hexosaminidase from RBL-2H3 cells is related to the time-effect relationship of released serotonin.

3.4 Practical application of the HPLC-ECD method

The amount of serotonin released by RBL-2H3 cells stimulated by different concentrations of schisandrin A and harpagoside was determined. As shown in Fig.4A, schisandrin A and harpagoside can cause RBL-2H3 cells to release serotonin. RBL-2H3 cells released serotonin in a dose-dependent with increasing concentrations of schisandrin A and harpagoside. These results correspond to the β-hexosaminidase release assay, as shown in Fig.4B. Our results demonstrate that schisandrin A and harpagoside can cause RBL-2H3 cells to release serotonin in a dose-dependent manner.

4. Conclusion

In this study, a new, sensitive HPLC-ECD method was established for the real time detection of serotonin released by RBL-2H3 cells simulated by potential allergens. This method provides for simple sample preparation, short analysis time, and high sensitivity, specificity, and reliability. The method was successfully used to determine the concentration of serotonin released by RBL-2H3 cells stimulated by different concentrations of schisandrin A and harpagoside. The results demonstrated that schisandrin A and harpagoside can cause RBL-2H3 cells to release serotonin in a dose-dependent manner. The study will contribute to the further use of evaluating potential allergens.

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Declaration of interest

The authors report no declarations of interest.

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Figure Legends

Fig.1 Effect of ECD detector voltage and sample volume on the signal intensity.

Fig.2 Typical chromatograms of different samples. (A) Actual sample, (B) blank samples with added serotonin, and (C) was blank sample (culture medium).

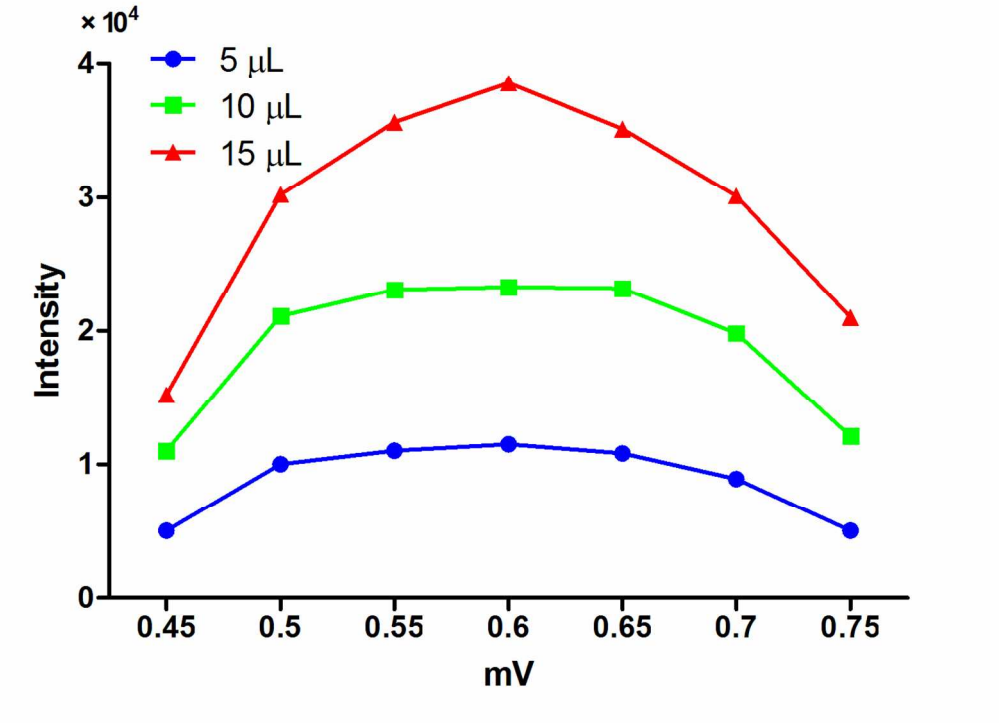
Fig.3 The time-effect relationship of released serotonin (A) and β-hexosaminidase (B) from RBL-2H3 cells which were stimulated by compound 48/80 by plotting the concentrations of the serotonin and β-hexosaminidase versus time (X-axis).

Fig.4 Released serotonin (A) and β-hexosaminidase (B) from RBL-2H3 cells which were stimulated by different concentrations of schisandrin A and harpagoside.

Table 1. Intra- and inter-day precision, and accuracy for the detection of released serotonin (n = 3 days, 5 replicates per day).

Table 2. Recovery of released serotonin determined by the HPLC-ECD method (n = 5).

Table 3. Stability of released serotonin samples determined by the HPLC-ECD method (n = 5).



Effect of ECD detector voltage and sample volume on the signal intensity.
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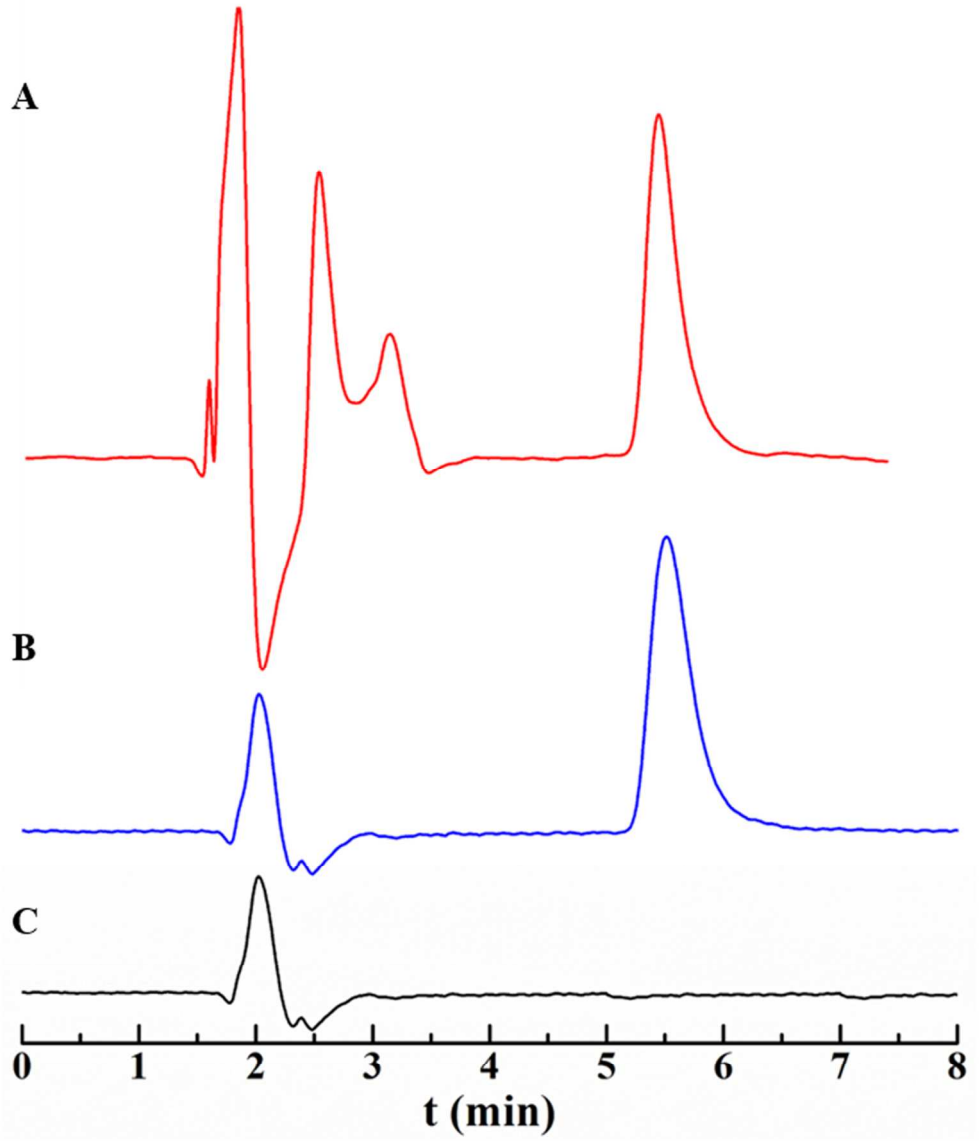

Typical chromatograms of different samples. (A) Actual sample, (B) blank samples with added serotonin, and (C) was blank sample (culture medium).
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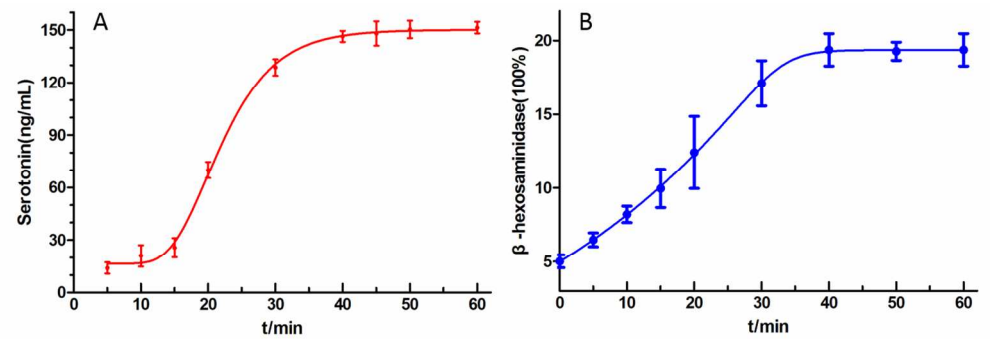
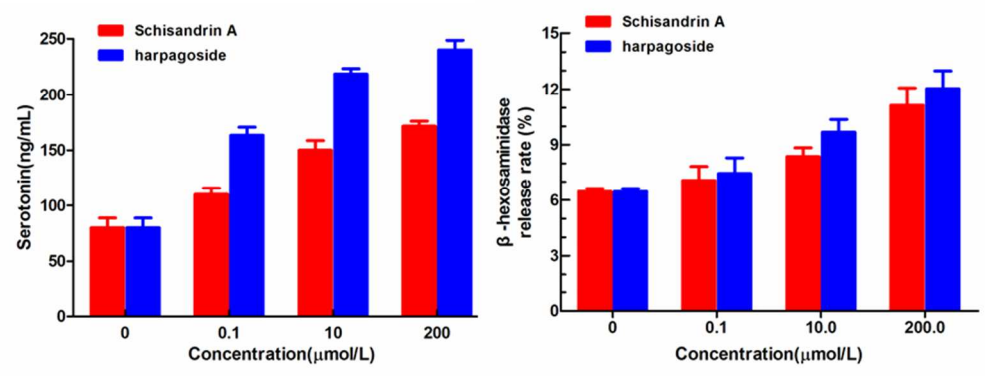


The time-effect relationship of released serotonin (A) and β-hexosaminidase (B) from RBL-2H3 cells which were stimulated by compound 48/80 by plotting the concentrations of the serotonin and β-hexosaminidase versus time (X-axis).
238x79mm (300 x 300 DPI)



Released serotonin (A) and β-hexosaminidase (B) from RBL-2H3 cells which were stimulated by different concentrations of schisandrin A and harpagoside.
76x29mm (300 x 300 DPI)

Table 1. Intra- and inter-day precision, and accuracy for the detection of released serotonin (n = 3 days, 5 replicates per day).

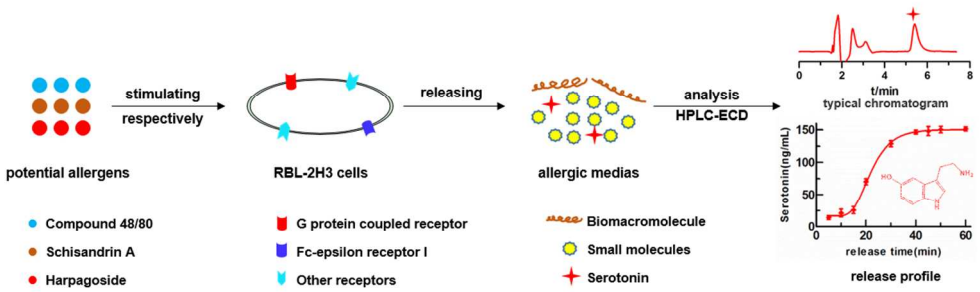
Analyte concentration (ng mL^{-1})	Intra-day (RSD %)	Inter-day (RSD %)	Accuracy (RE %)
150	1.35	2.02	-3.21
300	0.72	2.30	1.56
700	2.96	3.48	-0.89

Table 2. Recovery of released serotonin determined by the HPLC-ECD method (n = 5).

Statistical variable	Concentration levels (ng mL^{-1})		
	15	30	70
Recovery (Mean)	101.11	100.98	101.56
RSD (%)	1.35	0.72	2.28

Table 3. Stability of released serotonin samples determined by the HPLC-ECD method (n = 5).

Analyte concentration	Room temperature for 24 h		Three freeze–thaw cycles		Long term (30 days,-75 °C)	
(ng mL^{-1})	Precision (RSD %)	Accuracy (RE %)	Precision (RSD %)	Accuracy (RE %)	Precision (RSD %)	Accuracy (RE %)
15	10.85	-5.2	12.56	-6.1	9.65	4.7
30	11.23	-6.1	13.87	5.6	10.28	-6.2
70	10.96	4.9	11.82	4.9	8.89	5.4



An overview of the strategy for the evaluation of the potential allergens by detection of released serotonin from RBL-2H3 cells by the HPLC-ECD method.
105x32mm (300 x 300 DPI)