

Analytical Methods

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4 **1 A sensitivity HPLC-ECD method for detecting serotonin**
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6 **2 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 -chloroethylamine [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

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4 59 from RBL-2H3 cells, which are stimulated by potential allergens was urgently needed
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6 60 to evaluate potential allergens. There are many methods that have been reported to
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9 61 detect serotonin, such as HPLC-UV detection, HPLC-fluorescence detection, and
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11 62 enzyme-linked immune sorbent assay (ELISA) [12-14]. However, HPLC-UV
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14 63 detection and HPLC-fluorescence detection need derivatization, the process is
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17 64 complex [15, 16]. The ELISA method takes too long to perform and is not sensitive
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20 65 enough [17]. And there was a large amount of volatile salt in the sample after
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22 66 RBL-2H3 cells stimulated by allergens, it was not suitable for detection by mass
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25 67 spectrometry. In this study, we built a sensitive HPLC-ECD method for detection of
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28 68 released serotonin from RBL-2H3 cells stimulated by potential allergens. This method
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31 69 provides for simple sample preparation, short analysis time, and high sensitivity,
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34 70 specificity, and reliability compared with other technologies. The strategy of
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37 71 evaluation of the potential allergens by determination of released serotonin from
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40 72 RBL-2H3 cells by the HPLC-ECD method was as below. Compound 48/80, which
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43 73 was a positive drug to study allergies, was used to stimulate RBL-2H3 cells in the
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46 74 method development process [18]. A β -hexosaminidase release assay was performed
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49 75 by a biomedical method to verify the results. Schisandrin A and harpagoside, from
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52 76 traditional Chinese medicine, were previously reported as potential allergens [19,20].
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55 77 So, this method was used to detect the released serotonin from RBL-2H3 cells
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58 78 stimulated with different concentrations of schisandrin A or harpagoside, and expect
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60 79 to evaluate the sensitization of the two potential allergens.

80 **2. Materials and methods**

81 **2.1 Materials and reagents**

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

92 **2.2 Instruments and chromatographic conditions**

93 The HPLC system included a DGU-20A₃ degasser, two LC-20AD pumps, a
94 SIL-20A auto sampler, a CTO-20AC column oven, an LC-ECD-6A electrochemical
95 detector, and a Lab-solution workstation (Shimadzu Corporation, Kyoto, Japan). A
96 Chromolith® SpeedROD RP-18e column (50 mm×4.6 mm I.D., 2 μm) was used.
97 Isocratic elution with a flow of 1.0 mL/min was performed with two different mobile
98 phases. Mobile phase A was bufer composed of 0.1 M sodium dihydrogen phosphate
99 and 0.5 mM EDTA-2Na, pH 3.5 (sulfuric acid). Mobile phase B was methanol. The
100 mobile phase ratio was 95% mobile phase A, and 5% mobile phase B. The

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4 101 temperature of the column was maintained at 37 °C. The detection voltage of the
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6 102 electrochemical detector was set to 0.6V.
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9 103 **2.3 Standard and sample preparation**

10 104 Compound 48/80, serotonin, schisandrin A and harpagoside standard solutions
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12 105 were separately prepared in methanol (1 mg/mL). The solutions were freshly prepared
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14 106 each week and stored at -20 °C in darkness. When these standard solutions were used,
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16 107 they were diluted to suitable concentrations using freshly prepared mobile phase.
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19 108 Cells from exponentially growing cultures were used in the sample preparation
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21 109 for all experiments. RBL-2H3 cells were grown on 96-well plates (1×10^5 cells/well).
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23 110 Cells were cultured at 37 °C in a humidified atmosphere with 5% CO₂. The growth
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25 111 medium was replaced by modified-Tyrode's buffer (119 mM NaCl, 4.74 mM KCl,
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27 112 2.54 mM CaCl₂, 1.19 mM KH₂PO₄, 10 mM HEPES, 5 mM glucose, and 0.1% (w/v)
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29 113 BSA, pH 7.4). When cells reached approximately 80% confluence, they were treated
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31 114 with Compound 48/80, schisandrin A, and harpagoside at different concentrations.
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33 115 After 45 min of incubation at 37 °C, the 96-well plates were placed on an ice bath for
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35 116 10 min to stop the reaction. Then, the supernatant was transferred and filtered using a
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37 117 Millipore filter (0.45 μm), and the sample work solution was stored at -20°C in the
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39 118 dark until used for chromatography.
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50 119 **2.4 Optimization of the chromatographic conditions**

51 120 In order to shorten the analysis time, and get a good peak shape the concentration
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54 121 of buffer, the pH, and the ratio of buffer with methanol in the mobile phase were
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4 122 optimized. The voltage of the ECD detector and sample volume were also optimized
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6 123 to make the method more sensitive.
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9 124 **2.5 Method Validation**

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11 125 The selectivity, linearity, sensitivity, precision, accuracy, recovery, and stability
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13 126 of the HPLC-ECD method were validated according to the bioanalytical method
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15 127 validation guide published by the Food and Drug Administration (FDA) [21].
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19 128 2.5.1 Selectivity

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22 129 The selectivity was evaluated by analyzing blank samples (culture medium),
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24 130 blank samples with added serotonin, and actual samples, respectively.
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27 131 2.5.2 Linearity

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30 132 The calibration standards of serotonin were in the concentration range of
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32 133 10-1000 ng mL⁻¹. The concentrations 10 ng mL⁻¹, 50 ng mL⁻¹, 100 ng mL⁻¹, 200 ng
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34 134 mL⁻¹, 400 ng mL⁻¹, 800 ng mL⁻¹, 1000 ng mL⁻¹ were injected into the HPLC system,
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36 135 and the peak areas were recorded. Calibration curves were established by plotting the
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38 136 peak area versus serotonin concentration (X-axis).
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42 137 2.5.3 Sensitivity

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45 138 The sensitivity was shown by the lower limit of quantification (LLOQ). The
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47 139 LLOQ was defined as the lowest concentration of serotonin on the calibration curve
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49 140 with a relative standard deviation (RSD) lower than 20% and deviations from the
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51 141 nominal concentration within $\pm 20\%$ by five replicate analyses.
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55 142 2.5.4 Precision and accuracy

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4 143 The precision and accuracy were investigated (at low, middle and high
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6 144 concentrations) for samples in five replicates, which were prepared and analyzed on
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9 145 three consecutive days. RSD was used to evaluate the intra- and inter-day precision.
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11 146 To assess the accuracy, the relative error (RE) was calculated according to the formula:
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14 147 $RE\% = [(assayed\ value - nominal\ value) / nominal\ value] \times 100\%$.

17 148 2.5.5 Recovery

19 149 The recovery of serotonin was measured at three different concentration levels (n
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21 = 5 for each concentration).The recovery was calculated according to the formula:
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24 151 $Recovery = [(analyzed\ value - nominal\ value) / added\ value] \times 100\%$.

27 152 2.5.6 Stability

30 153 The stability was investigated by analyzing five replicates of the samples at three
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32 154 QC levels under different conditions, including 24 h storage at ambient temperature,
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34 155 three freeze/thaw cycles, and storage at -75 °C for 30 d. The samples were considered
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37 156 stable the average percentage concentration deviation was within 15% of the actual
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40 157 value.

43 158 **2.6 Time-effect relationship of released serotonin from RBL-2H3 cells**

45 159 The time-effect relationship of released serotonin from RBL-2H3 cells was
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48 160 investigated by injecting samples into the HPLC system after RBL-2H3 cells were
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51 161 treated with compound 48/80 treated (5–60 min, 5 min intervals, except the 25, 35,
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54 162 and 55 min time points. The concentration of serotonin at each of these time points
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57 163 was calculated. Then, the time-effect relationship of released serotonin from

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4 164 RBL-2H3 cells was established by plotting the concentrations of the serotonin versus
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6 165 time (X-axis).
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8 9 166 **2.7 β -hexosaminidase release assay**

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11 167 Another mediator, β -hexosaminidase, was detected to verify the time-effect
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13 168 relationship of an allergic mediator released by RBL-2H3 cells. β -Hexosaminidase is
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15 169 typically an enzyme marker of mast cell degranulation. RBL-2H3 degranulates upon
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17 170 treatment with multiple mediators, including histamine, serotonin, and
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19 171 β -hexosaminidase, in an almost identical manner, and the degranulation mechanism is
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21 172 the same as observed in primary mast cells and basophils [22]. In this study,
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23 173 RBL-2H3 cells were grown in 96-well plates (1×10^5 cells/well). The growth medium
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25 174 was replaced by modified- Tyrode's buffer. After 1 h incubation at 37 °C, cells were
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27 175 treated with compound 48/80 for 30 min, and then placed on an ice bath for 10 min to
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29 176 stop the reaction. At the end of the experiment, the supernatant was incubated with an
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31 177 equal volume of substrate solution (0.2 M citrate, 1 mM
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33 178 4-methylumbelliferyl-Nacetyl- β -D-glucosaminide, pH 4.5). Then 100 μ L of 0.2 M
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35 179 Tris-HCl buffer, pH 11 was used to rupture cells for 5 min. The absorbance of
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37 180 4-methylumbelliferone was measured with a Microplate Reader (Bio-rad, Hercules,
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39 181 USA) at 450 nm. To determine the total amount of β -hexosaminidase released, the
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41 182 remaining cells were lysed by treatment with assay buffer containing 1% (v/v) Triton
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43 183 X-100 prior to incubation with substrate using the same procedure as for
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45 184 determination of activity in the supernatant. The amount of β -hexosaminidase release
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4 185 was calculated by dividing the absorbance of the supernatant by that of the cell lyaste.
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6 186 The effects of different treatments on β -hexosaminidase are reported as percentage of
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9 187 control. The time-effect relationship of released β -hexosaminidase from RBL-2H3
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12 188 cells was investigated to verify the results of section 2.6.

14 189 **2.8 Practical application of the HPLC-ECD method**

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17 190 Schisandrin A and harpagoside are two potential allergenic components screened
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20 191 from traditional Chinese medicine in previous study [19,20]. In this study, the
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23 192 HPLC-ECD method was used to verify the sensitization of the two potential
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26 193 allergenic components. RBL-2H3 cells were grown in 96-well plates (1×10^5
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28 194 cells/well). The growth medium was replaced by modified- Tyrode's buffer. After 1 h
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30 195 of incubation at 37 °C, cells were treated with different concentrations of schisandrin
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33 196 A and harpagoside for 30 min and placed on ice bath 10 min to stop reaction. Then,
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36 197 the culture supernatant was collected and analyzed by HPLC-ECD as in section 2.2.
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38 198 In order to verify the results identified by the HPLC-ECD method, a
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41 199 β -hexosaminidase release assay was performed to verify the sensitization of the two
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44 200 potential allergens.

46 201 **3. Results and discussion**

48 202 **3.1 Optimization of the chromatographic conditions**

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51 203 In this study, a Chromolith® Speed ROD RP-18e column (50 mm \times 4.6 mm
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54 204 I.D., 2 μ m) was used to analyze serotonin released by RBL-2H3 cells. Satisfactory
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57 205 chromatographic behavior of serotonin was determined. Isocratic elution with a flow
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4 206 of 1.0 mL min⁻¹ was performed by using two different mobile phases. Mobile phase A
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6 207 was bufer composed of 0.1 M sodium dihydrogen phosphate and 0.5mM
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9 208 EDTA-2Na, pH 3.5 (sulfuric acid). Mobile phase B was methanol. The mobile
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11 209 phase ratio was 95% mobile phase A, and 5% mobile phase B. The retention time of
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14 210 serotonin was less than 6 min. The analysis time was relatively shorter than those in
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17 211 previous methods [23, 24]. The relationship between the voltage of the ECD detector,
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19 212 sample volume, and response intensity is shown in Fig.1. . The intensity was the
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21 213 highest when the voltage of ECD detector was 0.6 mV. In addition, when the sample
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23 214 volume was 10 µL, the response intensity was less volatile near 0.6 mV. Therefore,
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26 215 the voltage of ECD detector was fixed at 0.6 mV, and sample volume was 10 µL in the
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29 216 following study.

3.2 Method Validation

3.2.1 Selectivity

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38 219 No endogenous interference was observed at the retention time of serotonin (5.6
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40 220 min). Typical chromatograms of blank samples (culture medium), blank samples with
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43 221 added serotonin, and actual samples, are shown in Fig.2.

3.2.2 Linearity

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48 223 The typical calibration curves, correlation coefficients and linear ranges of
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50 224 serotonin were as follow. $A=344029C-1634$, A: peak area of serotonin, C:
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52 225 concentration of serotonin. The regression coefficient (r) was 0.9986. The linear range
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55 226 was from 10 ng mL⁻¹ to 1000 ng mL⁻¹.

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4 227 3.2.3 Sensitivity
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6 228 The lower limit of quantification of serotonin was always less than 10 ng/mL
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9 229 with good precision and accuracy, which indicated that the method was sufficiently
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11 230 sensitive. And this was more sensitivity than HPLC-UV method 32.5 ng/mL [25] and
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14 231 HPLC-fluorimetric detection 62 ng/mL [26].
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17 232 3.2.4 Precision and accuracy
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19 233 The results of accuracy and precision for intra-day and inter-day QC samples at
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22 234 three concentrations (150, 300, 700 ng mL⁻¹) were summarized in Table 1. The RSD
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25 235 was in the range of 0.72%–2.96% for inter-day precision, and in the range of
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27 236 2.02%–3.48% for intra-day precision. The REs were within ±3.21%. These results
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30 237 showed that the HPLC-ECD method was accurate and could reproducibly determine
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32 238 serotonin concentration.
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35 239 3.2.5 Recovery
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38 240 Recovery of the serotonin used in the HPLC-ECD method is shown in Table 2.
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41 241 At three concentration levels of serotonin (150, 300, 700 ng mL⁻¹), the recovery of
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43 242 serotonin was in the range of 100.98%–101.56%. And the RSD of recovery of
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45 243 serotonin was less than 2.28%. These results indicated that the recovery of the
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48 244 HPLC-ECD method for determining the serotonin concentration was acceptable.
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51 245 3.2.6 Stability
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54 246 The stability was investigated by analyzing five replicates of the samples at three
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56 247 QC levels under three different conditions, which the samples may experience,
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4 248 including 24 h storage at ambient temperature, three freeze/thaw cycles, and storage at
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6 249 -75°C for 30 d. The results shown in Table 3 demonstrated that the serotonin was
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9 250 stable after 24 h storage at ambient temperature (RSD% was less than 13.87%, RE%
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11 251 was within ± 6.1), three freeze/thaw cycles (RSD less than 11.23%, RE within ± 6.1 %),
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14 252 and storage at -75°C for 30 days (RSD less than 10.28%, RE within ± 6.2 %). These
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17 253 results indicated that the stability of the HPLC-ECD method for determining serotonin
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20 254 concentration was acceptable.

21 22 255 **3.3 Time-effect relationship of released serotonin from RBL-2H3 cells**

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25 256 The time-effect relationship of released serotonin from RBL-2H3 cells, which
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28 257 had been stimulated by potential allergens, is shown in Fig.3A. Between 5 min and 15
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30 258 min, the serotonin released by RBL-2H3 cells was stable, but the amount of released
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33 259 serotonin was low. Between 20 min and 30 min, the serotonin released by RBL-2H3
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36 260 cells increased as treatment time increased. Between 40 min and 60 min, the serotonin
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39 261 released by RBL-2H3 cells was stable, and the amount of serotonin released was high.
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42 262 In order to increase the sensitivity and reduce the system error, RBL-2H3 cells were
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45 263 stimulated for 45 min to release serotonin. This result was verified with a
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48 264 β -hexosaminidase release assay, and the results are shown in Fig.3B. Between 40
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51 265 min and 60 min, β -hexosaminidase release leveled off. So, the time-effect relationship
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54 266 of released β -hexosaminidase from RBL-2H3 cells is related to the time-effect
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57 267 relationship of released serotonin.

58 59 268 **3.4 Practical application of the HPLC-ECD method**

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4 269 The amount of serotonin released by RBL-2H3 cells stimulated by different
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6 270 concentrations of schisandrin A and harpagoside was determined. As shown in Fig.4A,
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9 271 schisandrin A and harpagoside can cause RBL-2H3 cells to release serotonin.
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11 272 RBL-2H3 cells released serotonin in a dose-dependent with increasing concentrations
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13 273 of schisandrin A and harpagoside. These results correspond to the β -hexosaminidase
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15 274 release assay, as shown in Fig.4B. Our results demonstrate that schisandrin A and
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19 275 harpagoside can cause RBL-2H3 cells to release serotonin in a dose-dependent
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22 276 manner.

277 **4. Conclusion**

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

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11 293 **Declaration of interest**

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14 294 The authors report no declarations of interest.

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9 398 Fig.1 Effect of ECD detector voltage and sample volume on the signal intensity.
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14 400 Fig.2 Typical chromatograms of different samples. (A) Actual sample, (B) blank
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16 401 samples with added serotonin, and (C) was blank sample (culture medium).
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22 403 Fig.3 The time-effect relationship of released serotonin (A) and β -hexosaminidase (B)
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24 404 from RBL-2H3 cells which were stimulated by compound 48/80 by plotting the
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26 405 concentrations of the serotonin and β -hexosaminidase versus time (X-axis).
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32 407 Fig.4 Released serotonin (A) and β -hexosaminidase (B) from RBL-2H3 cells which
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34 408 were stimulated by different concentrations of schisandrin A and harpagoside.
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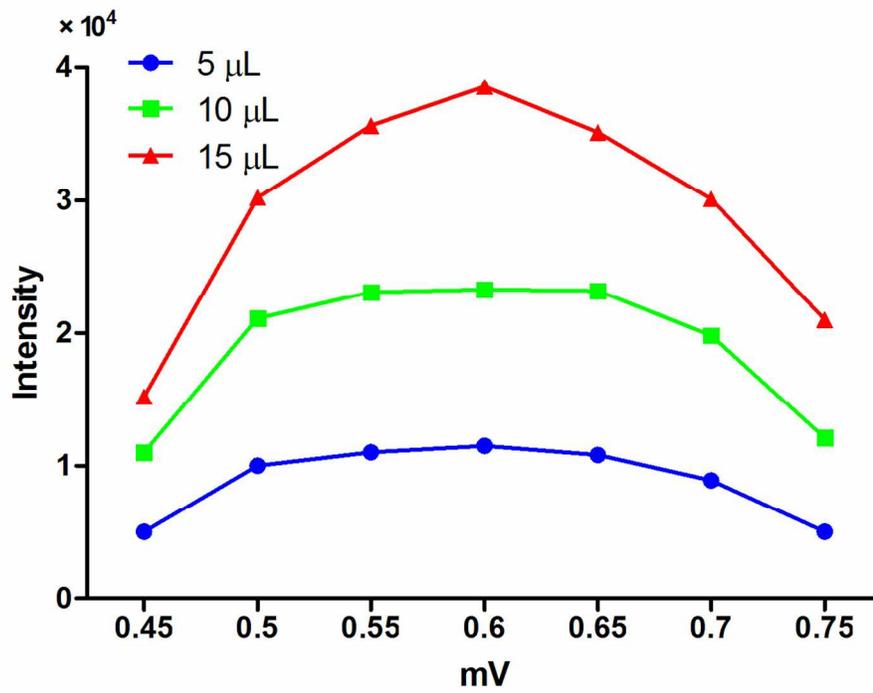
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40 410 Table 1. Intra- and inter-day precision, and accuracy for the detection of released
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42 411 serotonin (n = 3 days, 5 replicates per day).
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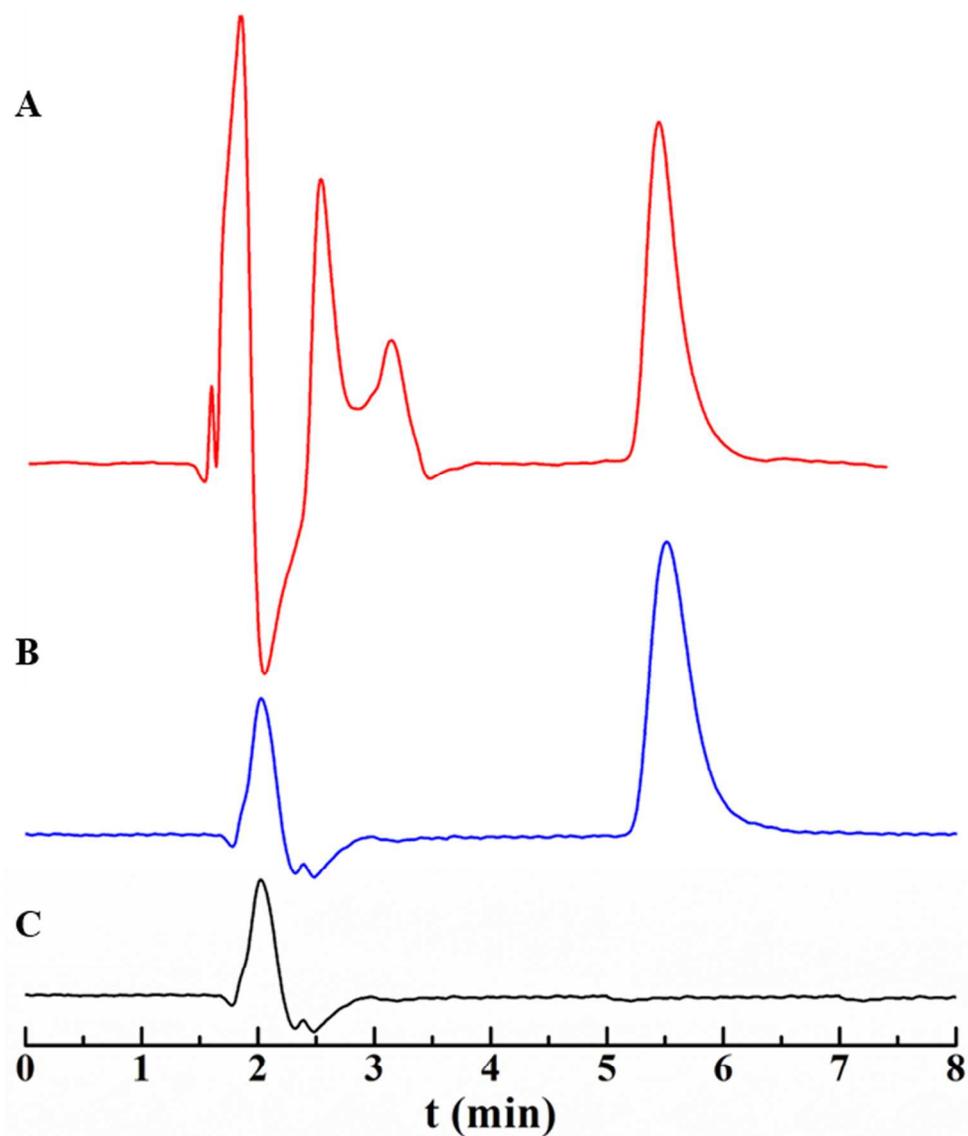
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47 413 Table 2. Recovery of released serotonin determined by the HPLC-ECD method (n =
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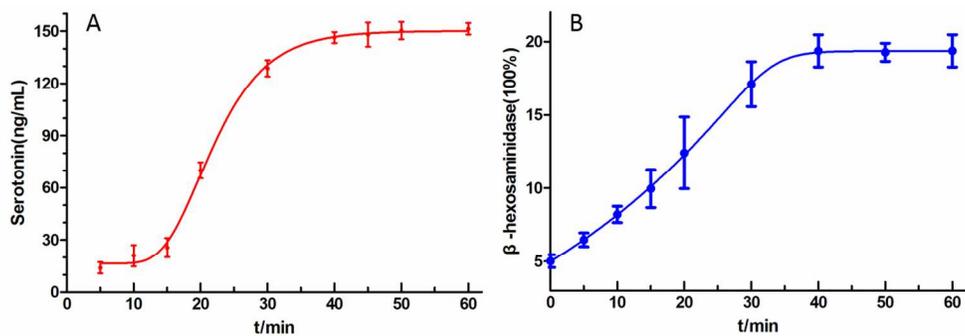
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53 416 Table 3. Stability of released serotonin samples determined by the HPLC-ECD
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55 417 method (n = 5).
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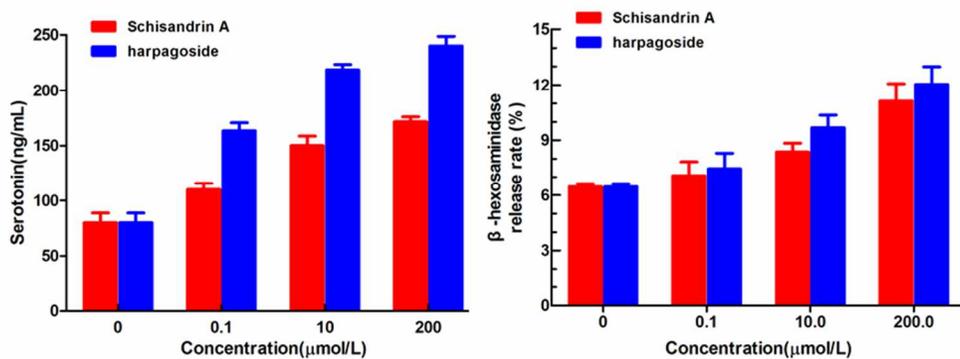
Effect of ECD detector voltage and sample volume on the signal intensity.
125x94mm (300 x 300 DPI)



Typical chromatograms of different samples. (A) Actual sample, (B) blank samples with added serotonin, and (C) was blank sample (culture medium).
112x133mm (300 x 300 DPI)



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

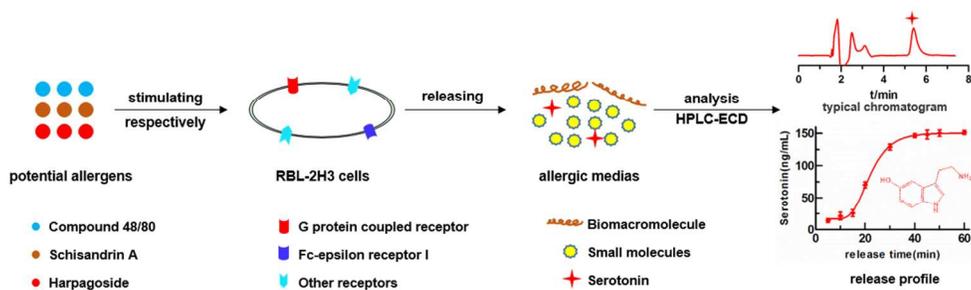
Table 2. Recovery of released serotonin determined by the HPLC-ECD method (n = 5).

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

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

Analyte concentration (ng mL ⁻¹)	Room temperature for 24 h		Three freeze–thaw cycles		Long term (30 days,-75 °C)	
	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)