Simultaneous determination of twelve biogenic amines in serum by high performance liquid chromatography

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In this study, we established a method for simultaneously determining twelve biogenic amines in serum by using reversed phase high performance liquid chromatography (RP-HPLC). The biogenic amines were first extracted from human serum by perchloric acid solution and derivatized by dansyl chloride. An ODS column was selected as separation column at 40 °C. The mobile phase solutions were consisted of A, 0.1 mol/L ammonium acetate and B, acetonitrile. A gradient elution was carried out with a flow rate at 1.0 ml/min. The results show that the detection limit for twelve biogenic amines ranged between 0.0621 and 0.628 μg/L. All the correlation coefficients were above 0.999. The linearity was over the range from 0.001 to 20 mg/L depending on individual biogenic amine. The intra-day and inter-day coefficients of variations were from 0.53% to 7.50% and from 1.10% to 7.25% respectively. The average analytical recovery in serum was from 92.02% to 107.65%. Moreover, the serum concentrations of tryptamine, tyramine and histamine in healthy females were found lower than that in healthy males significantly. The method is sensitive, convenient, and reliable, and suitable for simultaneous analysis of multiple biogenic amines in the clinical diagnosis and drug discovery.

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1. Introduction

In the post-genomic era the attention of proteomics and metabolomics has specifically been paid recently because it is believed that most physiologic or pathologic roles of the gene expression profile change ultimately are exerted through the dynamic change of protein of proteome and small molecules of metabolome. Therefore, the comprehensive analysis of the metabolome has a central role in the understanding of consequence of genetic or epigenetic differences. Indeed, systemic study of the unique fingerprints of small molecular profiles has an important significance not only in clinic medicine, but also in drug discovery [1].

Biogenic amines are a class of small molecules that are produced and metabolized in vivo, and play an important role in the course of pathology and physiology in human. It is well known that the putrescine, spermidine, spermine and cadaverine have close relationship with malignant tumors, and are index in many malignant tumors [2–4]. Histamine is an active amine complex, and may affect the reactions of many cells as a sort of chemical conductive substance in many states, such as anaphylaxis, dermatosis, phlogistic reactions, rheumatoid arthritis and many others [5]. Histamine may also affect the conduction of cerebral nerve and can produce the effect of sleepiness.

The research showed the relationship of histamine with colon cancer [6]. Tryptamine, 2-phenylethylamine, octopamine and tyramine are trace amines which are found to be the substances related to psychiatric condition [7,8]. In addition, 2-phenylethylamine, octopamine and tyramine are also shown to be pathogenic neurotransmitters of hepatic encephalopathy. Serotonin (5-HT), norepinephrine (NE) and dopamine (DA) are the most important monoamine neurotransmitters that regulate cardiovascular and neurological activity [9–15]. For example, 5-HT, NE and DA are found to be significantly importance in Parkinson’s disease, and be useful on the diagnosis of pheochromocytoma [16]. They are also the major mediators affecting psychiatric behavior, such as depression and other mental diseases [17,18]. Importantly the balance of these biogenic amines in vivo affects the function each other. Therefore, it is necessary to develop a simultaneous analysis of biogenic amine profile for the systemic monitoring their quantity in the blood or other body fluids.

Many methods were utilized for the determination of biogenic amines in serum or other body fluids, including fluorescence, ELISA, radioimmunoassay and high performance liquid chromatography (HPLC) [16–20]. Although the methods of fluorescence and ELISA are sensitive for detection, that cannot detect a dozen of biogenic amines simultaneously, and are monetary costly as well as time-consuming. Radioimmunoassay has the problems of radioisotope contamination besides of the disadvantage of fluorescence and ELISA. The HPLC has been used for the determination of biogenic amines, however, currently most methods with HPLC can only simultaneously detect a
few biogenic amines in serum or plasma [18–21]. Here we report a method with RP-HPLC to successfully measure twelve biogenic amines simultaneously in serum. We used steps of precolumn derivatization, gradient elution and ultraviolet detection, and found that the method established can be used for the needs of diagnosis and clinic research. We also found the sex difference in the serum concentration of tryptamine, tyramine, and histamine in the normal volunteers.

2. Materials and methods

2.1. Reagents and samples

HPLC grade acetonitrile and acetone were purchased from The Shanghai Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). The standard of DA was purchased from Fluka (Steinheim, Germany), and the standards of tryptamine, 2-phenylethylamine, putrescine, cadaverine, histamine, octopamine, 5-HT, tyramine, spermidine, NE, spermine and derivatization reagent dansyl chloride were purchased from Sigma (St. Louis MO). The HPLC grade water used was purchased from Merck (Darmstadt, Germany). The other reagents used in this work were of analytical grade and were obtained from The Shanghai Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

The standard solution was made by exactly weighing each standard of amine, and dissolving in ultra-pure water (HPLC grade). After dissolution, each standard was diluted to 1 g/L with ultra-pure water and was stored avoiding light at 4 °C as stock solution. The assay standards were freshly obtained by diluting the stock standard solutions with ultra-pure water for each assay. The derivatization reagent solution was dansyl chloride that was dissolved and diluted to 10 mg/ml with acetone, then stored at 4 °C, avoiding light.

The surplus serum samples that had been measured in routine analyses in the clinical chemistry laboratory at our hospital were collected and stored at −80 °C until analysis. All samples were anonymized and could not be linked to the donor’s identity.

2.2. Apparatus

The HPLC system consisted of a high-performance liquid chromatograph LC-10A (Shimadzu, Kyoto Japan), including LC-10AT binary pump, SIL-10AD automatic injection, SCL-10A controller, CTO-10A column oven, SPD-10A ultraviolet and visible detector, and CLASS-VP chromatogram analytical software.

2.3. Sample processing

To 0.5 ml of serum, 0.75 ml of 0.4 mol/L HClO₄ was added and then centrifuged at 3000 rpm for 10 min. The supernatant was aspirated into another test tube followed by adding 67.5 μl of 2.0 mol/L NaOH to mix. After further adding 150 μl of the saturated sodium bicarbonate and 1.0 ml of dansyl chloride, the mixture was reacted for 45 min at 40 °C, avoiding light. Finally 50 μl of 25% ammonia was added and incubated for 30 min before adding acetonitrile to 2.5 ml. The 2.5 ml of solution was filtrated by 0.20 μm needle shape colander before loading onto HPLC column for determination.

2.4. Standard treatment

The stock standard solutions were diluted with ultra-pure water. The concentration series of each standard were made at 0.001 mg/L, 0.005 mg/L, 0.01 mg/L, 0.05 mg/L, 0.1 mg/L, 1 mg/L, 5 mg/L, 10 mg/L, 20 mg/L and 40 mg/L, respectively. 0.5 ml of each standard, was operated as described above serum sample processing before loading to the HPLC column. The qualitative analysis was done by using the method of retention time, and the quantitative analysis was done by using the method of outside standardization. The calibration curves were evaluated by plotting the biogenic amines peak area values against the respective concentrations of biogenic amines standards.

2.5. Chromatographic conditions

The column was C₁₈ (150 mm×4.6 mm i.d., 5 μm particle size, Kromasil, Sweden) directly attached to a guard C₁₈ precolumn (13 mm×4.6 mm i.d.). The mobile phase solution A was 0.1 mol/L NH₄Ac and the mobile phase solution B was acetonitrile respectively. Prior to use, the mobile phase solutions were filtrated by 0.40 μm filter and then degassed with ultrasonic. Gradient elution was selected at 0–7 min, 45%A–50%A; 7–25 min, 50%A–10%A; 25–35 min 10%A–45% A; 35–45 min, 45%A. The temperature of the column was kept at 40 °C,
the mobile phase solutions were pumped at a flow rate of 1.0 ml/min, and 200 μL samples were injected. The wavelength of detection was 254 nm.

2.6. Statistics

The calculation of linearity relationship between peak areas and concentrations were adopted by Least-squares regression. The normal reference datum were shown by means of mean value ± standard deviation (mean ± s). The comparisons of average value between groups were adopted by T test.

3. Results and discussion

3.1. Detecting results of standard and serum

The chromatogram of twelve standard biogenic amines mixture was shown in Fig. 1, in which all of standard amine peaks appeared within 30 min, and there was no overlap between each standard amine. The detection peak of serum sample could be separated excellently within 30 min as shown in Fig. 2 chromatogram.

3.2. Extraction of serum sample

In our protocol we compared two extraction methods of serum samples by using trichloroacetic acid and perchloric acid respectively. We found better results were come from using perchloric acid for extracted reagent. The optimal concentration of perchloric acid for extraction was 0.4 mol/L compared with 1.0 mol/L and 2.0 mol/L. Too high concentration of perchloric acid will result in detected peaks disappearing nearly. Therefore, 0.4 mol/L of perchloric acid was used as extraction reagent.

3.3. Effect of reaction temperature on derivatization reaction

We found that if the reaction temperature was too low, the time of reaction needed was too longer, and the derivatization could not be completed. While the reaction temperature was too high, the polyamines could be decomposed. Moreover, the peak area could not be increased. Therefore, we found that the best suitable temperature was 40 °C, as shown in Supplement Fig. 3.

3.4. Effect of reaction time on derivatization reaction

We found that if the reaction time was too short, the derivatization could not be completed, while if the reaction time was too long, the peak area could not be increased. Our results showed that the best suitable time was about 45 min as shown in Supplement Fig. 4.

3.5. Concentration of dansyl chloride on derivatization reaction

We found that the high concentration of dansyl chloride will slightly increase the peak area. However, consistently with other report, this will also increase background peak area, consequently decreasing the sensitivity of determination, [19].

3.6. Effect of solution pH on derivatization reaction

In order to insure all of the biogenic amines detected can show peak separately, we found it must be carried out under weak base condition in that dansyl chloride reacts with biogenic amines. We found it is very important to control pH. In this experiment, we adjusted pH to 8.0–8.5 with sodium hydroxide and sodium bicarbonate. The effect of solution pH on derivatization reaction was carried out by changing the volume of sodium hydrosxide. All experimental operations were the same as above in Fig. 2 except pH. When 60 μL of 2.0 mol/L NaOH was added for decreasing pH to 6.5–7.0, the peak area and peak high of 5-HT, spermidine and spermine decreased evidently with higher background. If 75 μL of 2.0 mol/L NaOH was added for increasing pH to 9.0–9.5, the peak of 5-HT disappeared nearly, and the peak of tryptamine changed wider, while, the peak area and peak high of spermidine and spermine decreased evidently, meanwhile, the peaks of background and impurity increased evidently. Therefore, we concluded the condition used in the result of Fig. 2 was
the best, in which we chose the 67.5 μl of 2.0 mol/L NaOH to control the reaction pH.

3.7. Calibration curve

The calibration curves of twelve biogenic amines were constructed using the chromatographic peaks from injection of each standard at increasing concentrations in the 0.001–20 mg/L range. Least-squares regression was used for the calibration of the slope, intercept, and the correlation coefficient. There were good linearity relationships between peaks area and concentrations. All the correlation coefficients were above 0.999. The linearity were between the range from 0.001 to 20 mg/L for octopamine and spermidine, from 0.001 to 5 mg/L for histamine, from 0.005 to 20 mg/L for cadaverine, 2-phenylethylamine and NE, from 0.005 to 10 mg/L for tryptamine, putrescine and DA, from 0.005 to 5 mg/L for 5-HT, tyramine and spermine.

3.8. Detection limits experiment

Detection limits (D=2N/S, signal-to-noise ratio of 2 [22]) were calculated from each peak area in a chromatogram obtained with a standard solution, 0.217 μg/L for tryptamine, 0.155 μg/L for 2-phenylethylamine, 0.628 μg/L for putrescine, 0.0764 μg/L for cadaverine, 0.0621 μg/L for histamine, 0.129 μg/L for octopamine, 0.380 μg/L for 5-HT, 0.135 μg/L for tyramine, 0.162 μg/L for spermidine, 0.0773 μg/L for NE, 0.141 μg/L for DA, and 0.326 μg/L for spermine, respectively.

3.9. Stability of derivatization products

The stable experiment of serum sample derivatization products showed that the derivatization product of NE was not stable, and the concentration of NE must be analyzed at the intraday time after derivatization, the derivatization products of putrescine could stabilize for three days at 4 °C, avoiding light, the derivatization products of tyramine and spermidine could stabilize for two weeks at 4 °C, avoiding light. The other biogenic amines could stabilize for three weeks at 4 °C, avoiding light.

3.10. Precision experiment

The precision was tested using pooled serum taken from normally healthy subjects. A pooled serum was analyzed consecutive five times for intra-day, and was analyzed two times each day for successive ten days for inter-assay. The precision of the method, expressed as coefficients of variation (CV), ranged from 0.53 to 7.50% for intra-assay, and from 1.10 to 7.25% for inter-assay, respectively (Table 1).

### Table 1
Precision experiment of mixture serum sample analysis (μg/L serum)

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Intra-assay (n=5)</th>
<th>Inter-assay (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>CV</td>
</tr>
<tr>
<td>Tryptamine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-phenethylamine</td>
<td>2619.0</td>
<td>30.0</td>
</tr>
<tr>
<td>Putrescine</td>
<td>712.1</td>
<td>12.2</td>
</tr>
<tr>
<td>Cadaverine</td>
<td>206.0</td>
<td>4.1</td>
</tr>
<tr>
<td>Histamine</td>
<td>50.4</td>
<td>0.3</td>
</tr>
<tr>
<td>Octopamine</td>
<td>6.9</td>
<td>0.5</td>
</tr>
<tr>
<td>5-HT</td>
<td>57.7</td>
<td>2.9</td>
</tr>
<tr>
<td>Tyramine</td>
<td>259.0</td>
<td>2.30</td>
</tr>
<tr>
<td>Spermidine</td>
<td>446.0</td>
<td>2.4</td>
</tr>
<tr>
<td>NE</td>
<td>70.9</td>
<td>6.6</td>
</tr>
<tr>
<td>DA</td>
<td>66.1</td>
<td>0.7</td>
</tr>
<tr>
<td>Spermine</td>
<td>21.3</td>
<td>0.7</td>
</tr>
</tbody>
</table>

The standards were added to serum before sample cleanup and HPLC analysis. Each value is the average of three independent runs.

3.11. Recovery experiment

High and low concentration standard of biogenic amines were added into the serum samples known each biogenic amines concentration, respectively. The serum concentration of each biogenic amine was detected for three times, and the recovery rate of each standard was calculated. The average recovery rate ranged from 84.00% to 108.00% for low concentration and from 84.84% to 118.20% for high concentration respectively (Table 2).

### Table 2
Added standard recovery experiment of serum sample biogenic amines

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Sample added (μg/L serum)</th>
<th>Added</th>
<th>Actual (μg/L serum)</th>
<th>Av. recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptamine</td>
<td>259.0  250  500</td>
<td>250</td>
<td>260.0</td>
<td>103.2</td>
</tr>
<tr>
<td>2-phenethylamine</td>
<td>7.0</td>
<td>50</td>
<td>12.5</td>
<td>170.0</td>
</tr>
<tr>
<td>Putrescine</td>
<td>730.0  250  500</td>
<td>250</td>
<td>1000.0</td>
<td>120.0</td>
</tr>
<tr>
<td>Cadaverine</td>
<td>203.0  250  500</td>
<td>250</td>
<td>443.0</td>
<td>690.0</td>
</tr>
<tr>
<td>Histamine</td>
<td>49.6   250  500</td>
<td>250</td>
<td>318.0</td>
<td>589.6</td>
</tr>
<tr>
<td>Octopamine</td>
<td>6.8    250  500</td>
<td>250</td>
<td>217.0</td>
<td>507.0</td>
</tr>
<tr>
<td>5-HT</td>
<td>56.8   250  500</td>
<td>250</td>
<td>283.0</td>
<td>597.0</td>
</tr>
<tr>
<td>Tyramine</td>
<td>255.0  250  500</td>
<td>250</td>
<td>509.0</td>
<td>818.0</td>
</tr>
<tr>
<td>Spermidine</td>
<td>439.0  250  500</td>
<td>250</td>
<td>649.0</td>
<td>1030.0</td>
</tr>
<tr>
<td>NE</td>
<td>69.8   250  500</td>
<td>250</td>
<td>329.0</td>
<td>494.0</td>
</tr>
<tr>
<td>DA</td>
<td>66.1   250  500</td>
<td>250</td>
<td>326.0</td>
<td>587.0</td>
</tr>
<tr>
<td>Spermine</td>
<td>21.0   250  500</td>
<td>250</td>
<td>245.0</td>
<td>525.0</td>
</tr>
</tbody>
</table>

3.12. Range of normal reference value

With established protocol we detected the serum concentrations of twelve biogenic amines for 48 healthy volunteers (25 males, 23 females, mean age 35.82±10.12 years old.). The results were shown in Table 3. Our results showed that the normal reference value of polyamines and monoamines in human serum were similar to past reports [18,23,24], the trace amines have no comparable reference value in human serum because of the reference value have not been seen so far. On the other hand, we found that there was difference between males and females in the concentration of serum tryptamine, tyramine and histamine. The concentration of serum tryptamine in females was lower significantly than that in males (P<0.005). The concentrations of serum tyramine and histamine in females were also lower significantly, compared with that in males (P<0.05). However, there was no difference between males and females in the other serum biogenic amines. The recent research showed that there were direct or indirect relationship between tryptamine, tyramine and dumps [7,8,25], and the incidence of dumps was significantly higher in...
females than males [26]. Our results may suggest that the potential mechanism of higher incidence of dumps in female population could be lower serum tryptamine and tyramine levels in females than in males. At the same time, we found that the serum histamine level was lower in females than that in males as well. Further clinical research of these specific biogenic amines is warranted to correlate their clinic significance in females.

4. Conclusion

In conclusion, the present paper describes a RP-HPLC method for simultaneous determination of tryptamine, 2-phenylethylamine, putrescine, cadaverine, histamine, octopamine, 5-HT, tyramine, spermidine, NE, DA, and spermine in human serum. With this method, we first time found that the serum concentrations of tryptamine, tyramine and histamine in healthy females were significantly lower than that in healthy males. This new method is sensitive and reliable, and will be useful for diagnosing and tracing the therapeutic effect of a given medication on certain diseases. This method may also have multi-applications in drug discovery, such as monitoring the balance of biogenic amines simultaneously for a given drug candidate and a formula of Traditional Chinese Medicine. Upon the need, this method can be detected single biogenic amine or twelve biogenic amines and/or multiple biogenic amines in serum simultaneously.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.microc.2008.10.005.