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# Rapid and Reproducible Dibutylation Derivatization Coupled with Ultra-High Performance Liquid Chromatography-Tandem Mass Spectrometry for the Simultaneous Determination of Dopamine, Norepinephrine and 5-Hydroxytryptamine in Rat Brain Microdialysates

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## Abstract

A rapid and reproducible method has been developed for the simultaneous quantification of dopamine (DA), norepinephrine (NE), and serotonin (5-HT) in rat brain microdialysates. Derivatization was processed through reductive amination with butyraldehyde and sodium cyanoborohydride at 60˚C for 80 min incubation. Dibutylated monoamine neurotransmitters (MANTs) were directly analyzed with ultra-high-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). The chromatographic run time was shorter (2.1 min/sample) than previous studies [15] [18] [20]. Good linearity ($R^2 > 0.99$) was obtained for DA, NE and 5-HT in the range of 25 - 5000 pg/mL, 5 - 1000 pg/mL and 2.5 - 500 pg/mL, respectively. Acceptable precision (CV, 8.5% - 13.4%) as well as accuracy (recovery, 94.1% - 106.8%) could be acquired by analysis of six batches of quality control samples (QCs) at four different concentrations, which demonstrated the reliability and reproducibility of current method. This method was successfully applied to the simultaneous determination of DA, NE and 5-HT in rat brain microdialysates, where basal levels as well as elevated levels after dosing with amphetamine (AMPH) were quantified for all three MANTs. This study provides a simple and rapid way to analyze MANTs in the biofluid in the future.

**Keywords**

Derivatization, Microdialysis, Monoamine Neurotransmitters, LC-MS/MS

## 1. Introduction

Abnormalities in neurotransmitter synthesis, breakdown and transport represented a wide group of neurological syndrome [1]. Monoamine neurotransmitters (MANTs), including dopamine (DA), norepinephrine (NE) and serotonin (5-HT) have been proved to be highly correlated with a number of central nervous system symptoms, ranging from abnormal behavior [2], emotional fluctuation [3] [4], to a wide range of neurological diseases [1], such as depression [5] [6], Parkinson's disease [7], Huntingdon's disease [8] and Alzheimer's disease [9] [10] [11]. Therefore, *in vivo* detection of changes in MANTs in the brain was important for the elucidation of disease pathology, clinical diagnosis and evaluation of new treatment strategies of such disorders [1] [12].

Microdialysis was one of the few techniques that enabled continuous quantification of small molecules in the cerebrospinal fluid (CSF) of behaving animals [13], such as neurotransmitters and peptides. Slow perfusate flow rates were required to assure adequate relative recovery of an analyte *in vivo*, but would result in small sample collection volume or insufficient temporal resolution [13] [14]. In most of the previous studies, twenty to fifty μL of rat brain microdialysates were collected every 10 to 30 min at the perfusion flow rate of 1.0 - 2.0 μL/min [15]-[27], where corresponding relative recoveries were between 50% and 70% [14]. If the sample volume for bioanalysis could be further reduced, better temporal resolution or larger relative recovery would be acquired through shortening the sample collection interval or lowering the perfusate flow rate. Additionally, the remaining volume of the sample could be reserved for future reanalysis. The basal levels of DA, NE and 5-HT in rat brain microdialysate were typically in the range of tens to thousands of pictogram per milliliter [15]-[28]. Therefore, it is necessary to establish a sensitive bioanalytical method for monitoring low basal levels of MANTs with the small volume of rat brain microdialysate.

Various methods have been developed for the quantitative determination of MANTs. High performance liquid chromatography (HPLC) coupled with various detection techniques including electrochemical detection (EC) [23] [29] [30] [31], fluorescence-based detection (FL) [32] [33] [34] [35], chemiluminescence detection (CL) [36] [37] [38] and mass spectrometry-based detection (MS) [15]-[22] [24] [25] [26] [27] [28] [32] [39] have been presented. HPLC-EC offered relatively simple and sensitive methods, however, system robustness, reproducibility and specificity became challenges for this method [30] [31]. And, it was difficult for HPLC-EC to simultaneously determine DA, NE and 5-HT within a single run [15]. Pre-column derivatization coupled with HPLC-FL pro-

vided high sensitivity, but it required long separation time (7 - 40 min/sample) to effectively separate target analytes from interferences due to the complex nature of biological matrices [34] [35]. HPLC-CL could be carried out without pre-column derivatization, but this method was limited by its linear range and relatively long separation time (~10 - 20 min/sample) [36] [38]. HPLC-MS/MS was a highly specific analytical method due to the additional structural information provided by ion fragmentation patterns [15]. However, HPLC-MS/MS without pre-column derivatization either failed to provide enough sensitivity for detection basal levels of MANTs [21], or required long separation time (~30 min/sample) [22].

Chemical derivatization of the MANTs was widely applied to improve the LC-MS/MS detection, primarily through a reaction targeting the amine group and/or phenol-hydroxyl groups. The derivatization reagents utilized in previous studies could be generally characterized into three groups: acyl chlorides [16] [24] [28] [32] [39], sulfonyl chlorides [17] [18] [25] and aldehyde [15] [40] [41]. Acyl chlorides and sulfonyl chlorides reacted not only with amines but also with phenol-hydroxyl groups due to their strong nucleophilicity [18] [24]. Therefore, it was possible to detect high-level background noise if the derivatization was not sufficient. Additionally, the hydrolysis products of acyl- or sulfonyl chlorides [32] would affect the derivatization efficiency. Sometimes, extra steps such as synthesis of the derivatization reagents [16] [18] [27] [32] [39] or ultrasound-assisted dispersive liquid-liquid microextraction (UA-DLIME) [16] [17] [18] [20] [39] were performed before or after derivatization. Furthermore, acyl chloride and sulfonyl chloride were environmental unfriendly derivatization reagents. Derivatization where two methyl groups [41] or two ethyl groups [15] were added onto the amine groups via step-wise Schiff base reaction offered a fast and simple experimental procedure. Little interference and low-level background noise were observed [15], probably due to the relatively simple reaction pathway between aldehydes and the amine groups of MANTs.

Due to the highly hydrophobic nature of the derivatization reagents, long elution time was usually used with reverse phase liquid chromatography (RPLC) separation (~3.5 to ~30 min/sample) [15]-[22] [24] [25] [26] [27] [28] [32] [39]. The pharmaceutical industry had brought constant pressure on bioanalysts for shorter timelines, accompanied by the increased number of biological samples [42] [43]. The average run time of approximately 2 min/sample has become a norm both in the literature [43] and in our own experience in the environment of a contract research organization (CRO). In the search for better derivatization reagents, it has been shown that longer alkyl chain length on the derivatization reagent could improve the LC behavior and sensitivity of amino acids [44]. However, on the other hand, more hydrophobic derivatization reagent would lead to longer retention time and poor solubility in the aqueous phase, which meant that an additional liquid-liquid extraction (LLE) step would be required. Therefore, the type and the hydrophobicity of the derivatization reagent needed

to be carefully tuned.

In this work, we reported a rapid and reproducible method for the simultaneous determination of three MANTs, including DA, NE and 5-HT in rat brain microdialysate. Microdialysis samples were firstly derivatized with *n*-butyraldehyde and sodium cyanoborohydride at 60˚C for 80 min, and further analyzed with a rapid (2.1 min/sample) UPLC-MS/MS protocol. In a practical example, both basal and elevated levels of MANTs were successfully quantified over a 4-hour time course before and after administration of amphetamine (AMPH).

## 2. Experiment

### 2.1. Chemicals and Materials

Dopamine hydrochloride (DA·HCl), (-)-norepinephrine (NE), serotonin hydrochloride (5-HT·HCl), L-ascorbic acid, sodium cyanoborohydride, formic acid, acetonitrile, methanol, ammonium acetate, paraformaldehyde, benzaldehyde and butyraldehyde were purchased from Sigma-Aldrich (Sanit Louis, MO, USA). Stable-isotopic labeling internal standards
2-(3,4-dihydroxyphenyl)ethyl-1,1,2,2-$d_4$-amine hydrochloride,
(±)-norepinephrine-2,5,6,$\alpha,\beta,\beta$-$d_6$ hydrochloride and serotonin-$\alpha,\alpha,\beta,\beta$,-$d_4$ creatinine sulfate complex (free base forms of the three internal standards were abbreviated as $d_4$-DA, $d_6$-NE and $d_4$-5-HT, respectively) were supplied by C/D/N Isotopes Inc. (Quebec, Canada). Ethanol absolute was supplied by Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Artificial cerebrospinal fluid (aCSF) was purchased from Harvard Apparatus (Holliston, MA, USA). Water was purified with an ELGA water purification system (ELGA, Bucks, UK).

### 2.2. Preparation of Solutions

Neurotransmitter standards DA·HCl, NE, and 5-HT·HCl were dissolved in 25 mM L-ascorbic acid in purified water: MeOH (v; v, 4:1) to obtain 1 mg/mL solutions of DA, NE, and 5-HT (concentrations were calculated in their free base forms), respectively. One mg/mL solutions were further multi-step diluted with 25 mM L-ascorbic acid in purified water: MeOH (v; v, 4:1) to generate stock solutions of DA, NE and 5-HT with the concentrations of 200 ng/mL, 20 ng/mL and 20 ng/mL, respectively. Calibration standards (CSs) were obtained by serial dilution of each stock solution with aCSF to the following concentrations: DA (25 pg/mL, 50 pg/mL, 100 pg/mL, 250 pg/mL, 500 pg/mL, 1000 pg/mL, 2500 pg/mL and 5000 pg/mL), NE (5 pg/mL, 10 pg/mL, 20 pg/mL, 50 pg/mL, 100 pg/mL, 200 pg/mL, 500 pg/mL and 1000 pg/mL), and 5-HT (2.5 pg/mL, 5 pg/mL, 10 pg/mL, 25 pg/mL, 50 pg/mL, 100 pg/mL, 250 pg/mL and 500 pg/mL). Quality control samples (QCs) were prepared by diluting stock solutions with aCSF to four different concentrations (DA: 25 pg/mL, 75 pg/mL, 400 pg/mL and 4000 pg/mL; NE: 5 pg/mL, 15 pg/mL, 80 pg/mL and 800 pg/mL; 5-HT: 2.5 pg/mL, 7.5 pg/mL, 40 pg/mL and 400 pg/mL), representing the limits of detection (LODs), low, medium and high concentrations of the calibration range. In-

ternal standard (IS) solutions were prepared by individually dissolving salt forms of $d_4$-DA, $d_6$-NE, and $d_4$-5-HT in 25 mM L-ascorbic acid in purified water: MeOH (v;v, 4:1) to the concentration of 1 mg/mL (concentrations were calculated in their free base forms). Aliquot of each IS solution was further diluted with ethanol to obtain a mixture of $d_4$-DA, $d_6$-NE and $d_4$-5-HT with the concentrations of 5 ng/mL, 0.5 ng/mL and 0.5 ng/mL, respectively.

### 2.3. Derivatization Neurotransmitters with Butyraldehyde

Ten μL of CSs, QCs, or microdialysate sample was transferred into a vial containing 10 μL of IS mixture, 25 μL of sodium cyanoborohydride solution (1.5 mg/mL in ethanol) and 25 μL of butyraldehyde: ethanol (v: v, 2:98). The mixture was vortexed well and incubated in 60˚C water bath for 80 min, and was further analyzed by ultra-high performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS).

### 2.4. Method Validation

In order to evaluate the linearity and sensitivity of the proposed method, CSs of DA (25 - 5000 pg/mL), NE (5 - 1000 pg/mL) and 5-HT (2.5 - 500 pg/mL) were prepared and derivatized according to the method above (Section 2.2 and Section 2.3). The calibration curves were constructed by plotting the peak-area ratio (analyte/IS) against the spiked concentration of analyte in CSs with duplicate measurements using linear regression. The limits of detection (LODs) were defined as the concentration with the signal-to-noise ratio greater than 3 (S/N > 3). The accuracy and precision were assessed by the recovery and coefficient of variation (CV, %) of QCs at four different concentrations. QCs at each concentration level were paralleled prepared in six replications on the day of analysis. The recovery was calculated by (calculated analyte concentration/nominal analyte concentration) × 100%.

### 2.5. *In Vivo* Microdialysis Sampling

Male Sprague-Dawley rat (body weight 280 - 300 g) was provided by SLAC National Accelerator laboratory (CA, USA). The room temperature of animal lab was 20˚C -25˚C and humidity was 40% - 70%. Animal was allowed free access to food and water. Animal was anesthetized with pentobarbital sodium (50 mg/kg, intraperitoneal). BASi® BR-2 (2 mm membrane) brain microdialysis probes (Bregma polyacrylonitrile membrane, 30 KDa, 320 μm OD) (BASi corporate, IN, USA) was implanted with its tip at the upper limit of the striatum (coordinate in mm from Bregma: AP: +1.5; ML: +3.0; DV: −4.75). The probe was perfused with aCSF at 1.5 μL/min. After three days equilibration, microdialysis sampling was performed. Three consecutive microdialysate samples were collected at an interval of 20 min to determine the basal levels of DA, NE and 5-HT. Next, AMPH was intraperitoneally introduced at the concentration of 2 mg/kg. The concentration of AMPH, DA, NE and 5-HT were continuously monitored during a 220

min time course. Though 30 μL of microdialysate was collected every 20 min, only 10 μL was used for derivatization. The microdialysates were stored at −80˚C for future analysis. All the animal experiments were operated according to local ethical review and national legislation.

### 2.6. Sample Preparation for AMPH Analysis in rat Brain Microdialysis

An aliquot of 3 μL brain microdialysate was protein precipitated with 60 μL acetonitrile containing 100 ng/mL labetalol as IS. The mixture was vortexed well for 1 min and centrifuged at 13,000 rpm for 15 min at 4˚C. Twenty-five μL of supernatant was transferred into a new tube and the solvents were blow-dried under $N_2$. The residue was then reconstituted with 50 μL purified water. The solution was vortexed well and centrifuged at 13,000 rpm for 15 min at 4˚C. Seven μL of supernatant was injected for UPLC-MS/MS analysis of AMPH.

### 2.7. Ultra-High-Performance Liquid Chromatography/Tandem Mass Spectrometry

Neurotransmitter analysis was carried out with a QTRAP® 5500 mass spectrometer (Sciex, MA, USA) connected to an ACQUITY UPLC system (Waters, MA, USA) via an electrospray ionization (ESI) source. Separation protocol using an ACQUITY UPLC BEH C18 column (2.1 × 50 mm, 1.7 μm, Waters) was applied. The mobile phase gradient was set at 0.6 mL/min and 97% A for the first 0.2 min, and a linear gradient to 20% A at 1.2 min, and then to 2% A at 1.3 min, held 0.6 min, and returned to 97% A for 0.2 min to re-equilibrate the column, where A was acetonitrile:purified water (v: v, 5:95) containing 0.025% formic acid and 1 mM ammonium acetate and B was acetonitrile containing 0.025% formic acid and 1 mM ammonium acetate. The elution between 0.7 min and 1.0 min was flew into mass spectrometer, while the elution before 0.7 min and after 1.0 min were went to waste. Column temperature was 45˚C. The mass spectrometer was conducted in the positive mode with MRM transition. ESI settings were: source temperature was 600˚C, curtain gas was 40 psi, nebulizer gas pressure was 40 psi, auxiliary gas pressure was 60 psi and electrospray capillary voltage was 4.6 kV. MRM transition monitoring conditions were optimized for each compound and IS, and were summarized in Table 1. A 3.5 min separation protocol with a 3 μm-particle size, 2.1 × 100 mm ACE AQ column (Advanced Chromatography Technologies Ltd, Scotland) was applied for monitoring AMPH. The mobile phase gradient was set at 0.45 mL/min and 5% B for the first 0.3 min, and a linear gradient to 60% B at 2 min, and then to 95% B at 2.3 min, held 0.4 min, and returned to 5% B for 0.8 min to re-equilibrate the column, where A and B were consistent with the mobile phases utilized for derivatized MANTs. Column temperature was 45˚C. The mass spectrometer was conducted in the positive mode with MRM transition. ESI settings were: source temperature was 550˚C, curtain gas was 40 psi, nebulizer gas pressure was 50 psi and auxiliary gas pressure was 50 psi. MRM analysis parameters for AMPH were

**Table 1.** Parameters of derivatized DA, NE and 5-HT and corresponding IS in the MRM analysis. DP: declustering potential. CE: collision energy.

| Compound | Q1 Mass (*m*/*z*) | Q3 Mass (*m*/*z*) | Dwell Time (msec) | DP (eV) | CE (eV) | Retention time (min) |
|---|---|---|---|---|---|---|
| DA | 266.2 | 137.2 | 20 | 60 | 30 | 0.89 |
| NE | 282.2 | 264.3 | 20 | 70 | 25 | 0.86 |
| 5-HT | 289.1 | 142.1 | 20 | 80 | 23 | 0.93 |
| $d_4$-DA | 270.2 | 141.3 | 15 | 50 | 30 | 0.89 |
| $d_6$-NE | 288.2 | 270.3 | 15 | 70 | 25 | 0.86 |
| $d_4$-5-HT | 293.2 | 144.1 | 15 | 70 | 23 | 0.93 |

described as follows: Q1 Mass (136.2 Da), Q3 Mass (119.2 Da), dwell time (50 msec), declustering potential (DP) (40 eV) and collision energy (CE) (12 eV) and retention time (1.49 min).

## 3. Results and Discussion

### 3.1. Method Optimization

In this study, paraformaldehyde, benzaldehyde and butyraldehyde were investigated as derivatization reagents to react with DA, NE and 5-HT, respectively. Formaldehyde was reported as a common reagent to label amine groups [40] [41]. Dimethylated products were hard to retain on the RP column and eluted quite early together with other interference (data not shown), probably due to their strong polarity. Derivatization with excess benzaldehyde resulted in additional peaks right before the target compounds which would interfere their quantification (data not shown). Dibutylated DA, NE and 5-HT had good retention on the RP column, and the peak shape of these compounds were also satisfied. Therefore, we finally chose butyraldehyde as the derivatization reagent to perform reductive amination with DA, NE and 5-HT in this study (**Figure 1**).

The temperature and the duration of derivatization were optimized in order to obtain high conversion yield. Conversion yield was defined by Equation. (1), where *PA* represented the peak-area of target analyte at certain time point under certain reaction condition and $PA_{max}$ was the highest peak-area could be obtained under the same condition.

$$\text{Conversion yield}(\%) = \frac{PA}{PA\max} \times 100\% \quad (1)$$

The conversion yields for all three MANTs increased with time, and the rates were faster at higher temperatures (**Figure 2**). The highest conversion yields achieved after fourteen hours incubation under room temperature (**Figure 2(a)**) and reached maximum after 140 - 200 min at 37˚C (**Figure 2(b)**). DA, NE and 5-HT could achieve highest conversion yields within 80, 40 and 80 min at 60˚C, respectively (**Figure 2(c)**). Finally, incubation of MANTs with butyraldehyde at 60˚C for 80 min was chosen as the reaction condition for derivatization. The

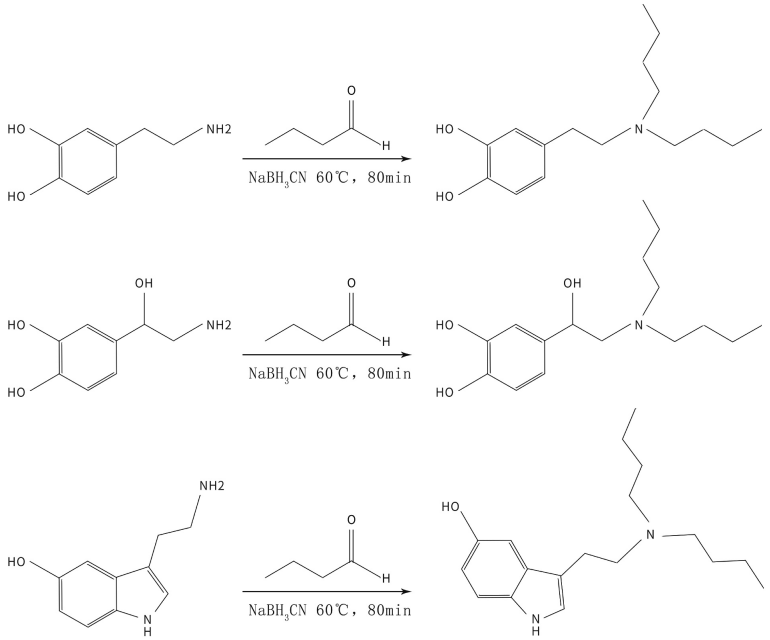


**Figure 1.** Reductive amination of DA, NE and 5-HT with butyraldehyde.

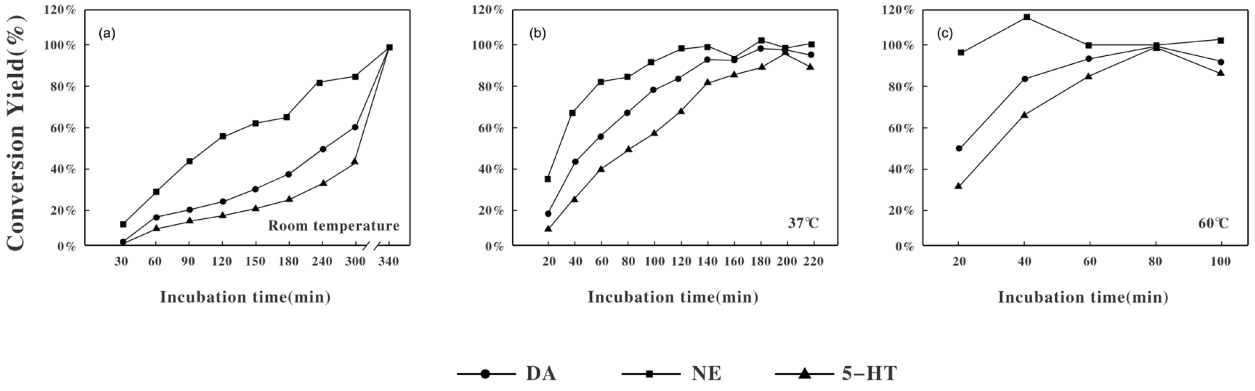


**Figure 2.** Conversion yield of DA, NE and 5-HT into corresponding dibutylated derivatives under: (a) Room temperature; (b) 37˚C and (c) 60˚C.

products were stable at 10˚C after 26 hours of storage in the UPLC autosampler (data not shown). Different volumes of the derivatized MANTs (6 - 10 μL) were injected into UPLC/MS/MS system to investigate the effect of injection volume on the detection of MANTs. The results showed that the optimal peak shape could be obtained when the injection volume was 6.5 μL. Lower injection volume would reduce the intensity of the signals, while higher injection volume would result in split peaks (data not shown).

In general, reductive amination of DA, NE and 5-HT with butyraldehyde at 60˚C for 80 min provided a simple way to convent MANTs into corresponding dibutylated derivatives with high conversion yield. The current method was simple and did not require complex sample preparation steps, such as synthesis of the derivatization reagents or an UA-DLIME procedure. The optimal chro-

matographic peak shape of the derivatized products could be obtained with the injection volume of 6.5 μL.

### 3.2. Method Validation

The investigated linearity ranges of DA, NE and 5-HT were 25 - 5000, 5 - 1000 and 2.5 - 500 pg/mL, respectively. Good linearity correlations were obtained for the three MANTs with the coefficient of determination ($R^2$) all above 0.99 (**Table 2**). The LODs (S/N > 3) for DA, NE and 5-HT were 25, 5 and 2.50 pg/mL, respectively (**Table 2**). Chromatograms of derivatized DA and their corresponding internal standards were presented in **Figure 3**. It was reported that the basal levels of MANTs were around tens to thousands of pictograms per milliliter in rat brain microdialysate [15]-[28]. Therefore, the above results suggested that current method had adequate sensitivity for simultaneous determination of DA, NE and 5-HT in rat brain microdialysate. The intra-day coefficient of variance (CV, %) of six batches of QCs were all below 13.4%, 12.0% and 8.5% for DA, NE and 5-HT, suggesting good precision and reproducibility of the proposed method. Recovery of all three compounds at different concentrations were within ± 15% of spiked amount. All validation data suggested that present method was reliable and reproducible, and could be applied to accurate quantification of DA, NE and 5-HT in the aCSF matrix.

### 3.3. Application of Proposed Method to Rat Brain Microdialysate

The developed method was applied to simultaneously monitor DA, NE and

**Table 2.** The linearity range, LODs (limits of detection, S/N > 3), $R^2$ (coefficient of determination), precision (coefficient of variation, CV, %) and accuracy (recovery, %) of the current method. Both the precision and the accuracy were obtained from the analytical results of six batches of QCs.

| Analyte | Linearity Range (pg/mL) | LODs (pg/mL) | $R^2$ | Spiked amount (pg/mL) | Precision (CV, %) | Accuracy (recovery %, mean ± SD) |
|---|---|---|---|---|---|---|
| DA | 25 - 5000 | 25 | 0.9960 | 25 | 13.4 | 103.3 ± 13.9 |
| | | | | 75 | 6.4 | 95.1 ± 6.1 |
| | | | | 400 | 2.0 | 100.0 ± 2.0 |
| | | | | 4000 | 2.0 | 104.5 ± 2.0 |
| NE | 5 - 1000 | 5 | 0.9966 | 5 | 12 | 102.8 ± 12.4 |
| | | | | 15 | 10.1 | 88.9 ± 9.0 |
| | | | | 80 | 4.5 | 97.4 ± 4.4 |
| | | | | 800 | 2.6 | 94.1 ± 2.5 |
| 5-HT | 2.5 - 500 | 2.5 | 0.9928 | 2.5 | 8.5 | 95.5 ± 8.1 |
| | | | | 7.5 | 5.8 | 102.6 ± 5.9 |
| | | | | 40 | 8.3 | 106.8 ± 8.9 |
| | | | | 400 | 5.2 | 101.8 ± 5.3 |

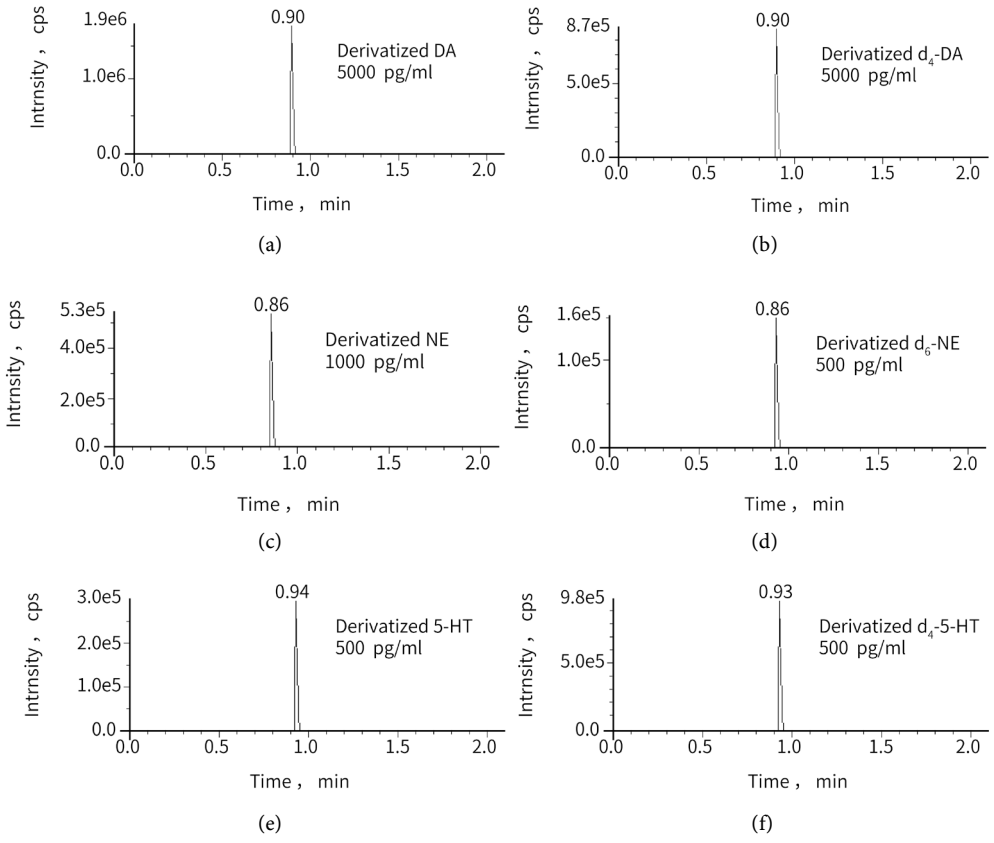


**Figure 3.** The chromatograph of (a) Derivatized DA; (b) Derivatized $d_4$-DA; (c) Derivatized NE; (d) Derivatized $d_6$-NE; (e) Derivatized 5-HT and (f) Derivatized $d_4$-5-HT, respectively.

5-HT in SD rat brain microdialysates for a course of 220 min (**Figure 4**). Thirty μL of the microdialysate was collected at each time point, and only 10 μL was used for derivatization. Three consecutive microdialysates were collected before intraperitoneal injection of AMPH to monitor basal levels of MANTs. The basal concentration of DA (**Figure 4(b)**), NE (**Figure 4(c)**), and 5-HT (**Figure 4(d)**) were in the ranges of 143-159 pg/mL, 5.0-7.4 pg/mL and 14.7-25.0 pg/mL, respectively. Significant concentration increase was observed for all MANTs after AMPH administration. DA (**Figure 4(b)**), NE (**Figure 4(c)**) and 5-HT (**Figure 4(d)**) reached highest concentration after 40 min, 40 min, and 60 min injection of AMPH, respectively, and subsequently decreased with time.

## 4. Discussion

### Comparison with Previous Studies

This study presented a dibutylation derivatization coupled with a rapid UPLC-MS/MS (2.1 min/sample) to simultaneous determination of DA, NE and 5-HT. The acceptable precision (8.5% - 13.4%, CV) and accuracy (recovery, 94.1% - 106.8%) demonstrated the reliable and reproducibility of the current method. The derivatization procedure was simple and easy to handle without any extra steps, such as synthesis of derivatization reagent in advance [16] [18]

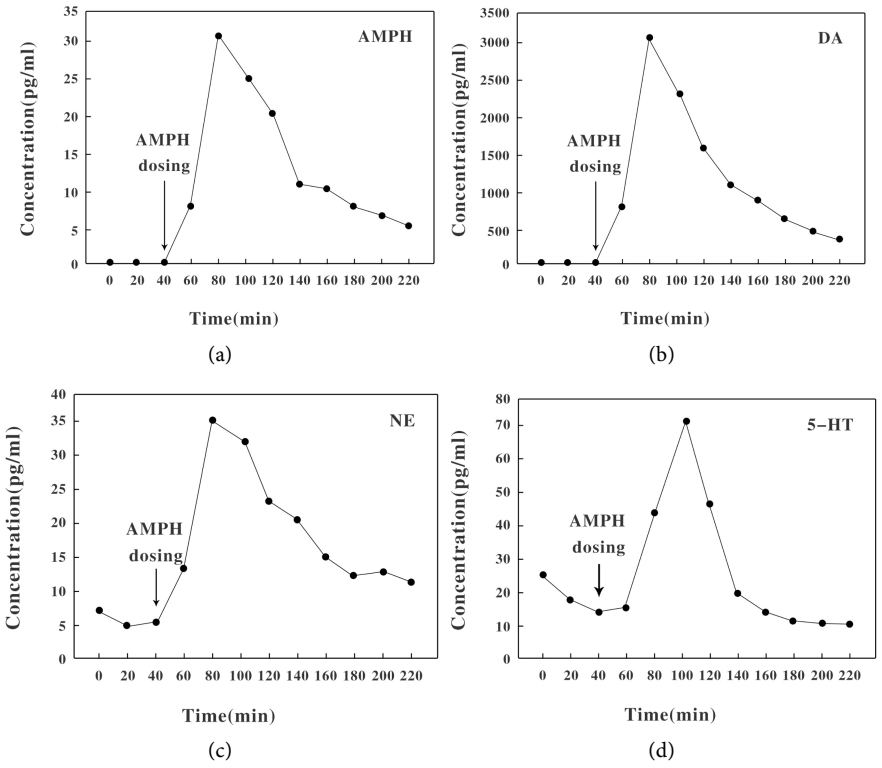


**Figure 4.** Concentration of AMPH, DA, NE and 5-HT in the brain microdialysate of a SD rat. AMPH was intraperitoneally injected at 40 min. AMPH: amphetamine. DA: dopamine. NE: norepinephrine. 5-HT: serotonin.

[27] [32] [39] or an UA-DLIME procedure after derivatization [16] [17] [18] [20] [39]. Direct measurement of DA, NE and 5-HT without chemical derivatization resulted in poor sensitivity, which was not sensitive enough to evaluate the basal levels of MANTs in rat brain microdialysate [21]; or such method could only analyze DA, and required LC separation time as long as 30 min [22]. The LODs of NE and 5-HT after derivatization with benzoyl chloride were relatively high (~34 pg/mL and 18 pg/mL, respectively), and UPLC-MS/MS run time was four time longer than current study [28]. In our own effort to reproduce this method, very high background noise was observed (data not shown). Diethyl reductive amination approach offered adequate sensitivity (5 - 10 pg/mL) and shorter analysis time (3.5 min/sample) [15], however, the results were difficult to reproduce when the same procedure was tried in author's lab. Sensitivity was higher if MANTs were derivatizied with LRSC or $d_0$-MASC (<0.1 pg/mL), however, both methods needed relatively long analytical run time (7 min and 5 min, respectively) and $d_0$-MASC needed to be synthesized in advance [17] [18]. Although our method did not provide the lowest LODs, it was adequate to quantify basal levels of the three MANTs in the rat brain microdialysate (tens to thousands of pictograms per milliliter) [15]-[28]. Last but not least, butyraldehyde was more environmental friendly reagent compared with acyl chloride and sulfonyl chloride.

Low perfusion rate was a guarantee of an analyte's recovery *in vivo*, but re-

sulted in small sample volume or poor temporal resolution [13] [14]. Quantification of low basal level of MANTs with a small volume of microdialysate became a big challenge for the bioanalysts. In most previous studies, more than twenty μL of the microdialysate were utilized for derivatization or direct analysis by the instruments [15]-[21] [23] [25] [27]. If the requirement of sample volume was reduced, better temporal resolution could be obtained [13] [14] and the rest portion of the sample could be reserved for the future reanalysis. In present study, one third of the collected microdialysate (10 μL out of 30 μL) was applied for derivatization which was smaller than most of the previous studies [15]-[21] [23] [25] [27]. Song *et al.*, provided a method using 5 μl of microdialysate [28] for derivatization, but their LODs of NE and 5-HT were relatively high (~34 pg/mL and 18 pg/mL) compared with current method.

Short chromatography run-time was another important evaluation criterion for a good bioanalytical method, which was an important consideration for improving efficiency of a lab. The UPLC-MS/MS run time of present study was 2.1 min/sample, which was the shortest compared with previous studies (~3.5 min~30 min) [15]-[22] [24] [25] [26] [27] [28] [32] [39]. The derivatized MANTs all eluted between 0.7 min and 1.0 min, and only this portion of the LC eluent was directly flew into the mass spectrometer, while the LC eluent at other time was went to waste. Current method could be easily adapted to a dual-arm high-throughput autosampler (Apricot Design Dual Arm, ADDA) coupled with LC-MS/MS technique system [45] [46]. While operated under gradient mode, two liquid chromatography systems could be incorporated into one, while one LC system performed analyte separation and the other LC system executed functions such as needle washing, column equilibration and sample injection. A switch valve determined the eluent of which LC system went into the mass spectrometer for data acquisition. If current method would be successfully transferred to ADDA-LC-MS/MS platform, the chromatography run time could be further shorten to half and the efficiency would be double in the future.

## 5. Conclusion

In this work, a simple dibutylation derivatization method coupled with a rapid UPLC-MS/MS was developed for the simultaneous determination of DA, NE and 5-HT. Derivatization was easy to handle, which was performed with butyraldehyde and sodium cyanoborohydride in 60˚C water bath for 80 min incubation. A rapid 2.1 min UPLC-MS/MS protocol was established, which was shorter than previous studies. This method was successfully applied to detect the basal and elevated levels of DA, NE and 5-HT with a small volume of rat brain microdialysate (10 μL). Acceptable validation results suggested that this method was reliable and reproducible. Present study provided a solid foundation for the future development of MANT analysis in the body fluids.

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