Anal. Chem. **2008,** *80,* 9195–9203

Diethylation Labeling Combined with UPLC/MS/MS for Simultaneous Determination of a Panel of Monoamine Neurotransmitters in Rat Prefrontal Cortex Microdialysates

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The primary challenge associated with the development of an LC/MS/MS-based assay for simultaneous determination of biogenic monoamine neurotransmitters such as norepinephrine (NE), dopamine (DA), serotonin (5-HT), and normetanephrine (NM) in rat brain microdialysates is to improve detection sensitivity. In this work, a UPLC/MS/MS-based method combined with a diethyl labeling technique was developed for simultaneous determination of a panel of monoamines in rat prefrontal cortex microdialysates. The chromatographic run time is 3.5 min/sample. The limits of detection of the UPLC/MS/MS-based method for NE, DA, 5-HT/ and NM, with/without diethyl labeling of monoamines, are 0.005/0.4 (30/2367 pM), 0.005/0.1 (33/653 pM), 0.005/0.2 (28/1136 pM), and 0.002/0.2 ng/mL (11/1092 pM), respectively. Diethyl labeling of amino groups of monoamines affords 20−100 times increased detection sensitivity of corresponding native monoamines during the UPLC/MS/MS analysis. This could result from the following: (1) improved fragmentation patterns; (2) increased hydrophobicity and concomitantly increased ionization efficiency in ESI MS and MS/MS analysis; (3) reduced matrix interference. This labeling reaction employs a commercially available reagent, acetaldehyde-d_4, to label the amine groups on the monoamines via reductive amination. It is also simple, fast (~25-min reaction time), specific, and quantitative under mild reaction conditions. Data are also presented from the application of this assay to monitor the drug-induced changes of monoamine concentrations in rat prefrontal cortex microdialysate samples followed by administration of SKF 81297, a selective D_1 dopamine receptor agonist known to elevate the extracellular level of the neurotransmitters DA and NE in the central nervous system.

Simultaneous determination of changes in basal levels of biogenic monoamines such as serotonin (5-HT), dopamine (DA), norepinephrine (NE), and normetanephrine (NM) in the rat brain is an important step in the process of discovery and evaluation of new drugs designed to treat disorders of the central nervous system, which are related to these biogenic monoamine neurotransmitters.[1] In vivo microdialysis is currently one of the main techniques used to continuously monitor the effects of drugs that act on extracellular pools of rat brain monoamines and their metabolites.[2] However, basal levels of monoamines in rat brain microdialysates are typically in the low picogram per milliliter range, which makes analytical measurement a challenge.

Various methods for determination of 5-HT, DA, NE, and their metabolites have been reported. [3−10] Among them, the most frequently used methods for monitoring NE, DA, 5-HT, and their metabolites are high-performance liquid chromatography coupled with electrochemical detection (HPLC-EC)[3,6] and HPLC coupled with fluorescence detection (HPLC-FL).[8−10] HPLC-EC offers a relatively simple and highly sensitive analytical method. However, it is difficult to use this technique for simultaneous determination of all four monoamines in microdialysis samples.[8] Alternatively, HPLC-FL provides a means of simultaneous determination of monoamines in mircodialysis samples. In addition to the need for multiple sample treatment steps, the lengthy HPLC run time (more than 40 min/analytical run) is a drawback of this technique when it is used for simultaneous determination of a panel of monoamines in microdialysis samples.[9,10] Recently, Jung and coworkers have reported a novel approach for simultaneous determination of biogenic monoamines in rat brain striatum within a

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10.1021/ac801339z CCC: $40.75
Published on Web 10/25/2008

short analysis time of less than 10 min.[11] Capillary HPLC coupled with photoluminescence following electron transfer (PFET) offers detection limits comparable to those of HPLC-EC and HPLC-FL and overcomes the shortcomings associated with HPLC-CE (e.g., electrode fouling) and HPLC-FL (e.g., need for a pretreatment step before sample injection).

In contrast to HPLC-CE, HPLC-FL and capillary HPLC-PFET, in which analytes can only be identified by retention time matching, LC/MS/MS can provide high specificity due to the additional structure information obtained using tandem mass spectrometric analysis. However, without a pretreatment step, LC/MS/MS cannot offer adequate sensitivity for simultaneous measurement of monoamines in rat brain microdialysates. A recently reported LC/MS/MS-based assay by Hows and co-workers was not able to determine basal levels of NE and 5-HT in rat brain microdialysis due to its poor sensitivity.[12]

Chemical derivatization of amine groups on amino acids with different chemical reagents has been shown to enhance amino acid detection by ESI MS analysis.[13−16] Differential isotopic dimethyl labeling of N-terminal peptides with d(0)- and d(2)-, or d(0), ^{12}C-, and d(2), ^{13}C-formaldehyde combined with LC-ESI or LC-MALDI was used successfully in quantitative proteomics.[17−20] N-Terminal dimethyl labeling was also reported for peptide sequencing in combination with database searching and de novo peptide sequencing of neuropeptides directly from tissue extract without any genomic information.[21−23] More recently, Guo and co-workers reported an approach for quantitative profiling of amine-containing metabolites in biological samples using LC-ESI MS in conjunction with stable-isotope dimethyl labeling.[24] The labeling is carried out by using reductive amination chemistry.[25] This chemical labeling has been reported to be simple, fast (less than 10-min reaction time), specific, and provide high yields under mild reaction conditions when used to label peptides and amine-contained metabolites.[17,24]

In this work, we report the successful combination of UPLC/MS/MS with diethyl labeling of each primary amine group on monoamine neurotransmitters, via reductive amination, for simultaneous measurement of a panel of monoamine neurotramitters in rat prefrontal cortex microdialysates. A practical example of the developed method is presented in which elevated levels of NE, DA, and 5-HT, NM were detected over a 4.5-h time course in the rat prefrontal cortex microdialysates following administration of SKF-81297, a selective D_1 dopamine receptor agonist known to elevate the extracellular level of the neurotransmitter DA and NE in the central nervous system.

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EXPERIMENTAL SECTION

Chemicals and Reagents. (±)-Normetanephrine- α, *β*, *β*-d3 (d3-NM) HCl (98.7 atom % D), serotonin-a,a,b,b-d_4 (d4−5-HT) creatinine sulfate complex, and 2-(3,4-dihydroxyphenyl)ethyl-1,1,2,2-d_4-amine (d4-DA) HCl were purchased from C/D/N Isotopes Inc. (Quebec, Canada). Sodium acetate, acetic acid, ammonium acetate, HPLC-grade water, and methanol were obtained from Mallinckrodt Becker (Phillipsburg, NJ). Formic acid (96%) was analytical grade and obtained from Acros Organics. Perfusion Fluid CNS was purchased from CMA Microdialysis Inc. (North Chelmsford, MA). All other chemicals and reagents were purchased from Sigma (St. Louis, MO) unless otherwise stated.

Reductive Amination. The derivatization of NE, DA, 5-HT, and NM was adopted and modified from Guo et al.[24] To profile the derivatized monoamines, 10 μL of acetate buffer (1 M, pH 5) and 20 μL of cyanoborohydride coupling buffer (containing 0.02 M sodium phosphate, pH 7.5, containing 0.2 M sodium chloride and 3.0 g/L sodium cyanoborohydride) was added to four different 1.5-mL tubes, each containing 40 μL of 0.4 μg/mL NE, DA, 5HT, and NM dissolved in HPLC-grade water, repectively. Then, the mixture solution was vortexed and another 10 μL of 20% acetaldehyde-d_4 was added to each tube. The final mixture solution was vortexed and incubated at 37 °C for 25 min, followed by LC/MS and LC/MS/MS analysis as described in the following sections. To examine the effect of the concentration of cyanoborohydride on the labeling efficiency, 0, 5, 10, 20, 30, and 40 μL of cyanoborohydride coupling buffer were added into different wells of a 96-well plate. Each well contained 30 μL of standard solution, with a concentration of 1 ng/mL for each monoamine of interest, which was prepared by diluting the stock solution using artificial CSF/preservative solution (5 μM EDTA and 100 μM citric acid) (5:1, v/v). Then the solution in each well was brought up to the same volume by adding 35, 30, 20, 10, and 0 μL of artificial CSF/preservative solution (5 μM EDTA and 100 μM citric acid) (5:1, v/v), followed by the addition of 20 μL of 1 M acetate buffer and 10 μL of 25% acetaldehyde-d_4. The resulted mixture was vortexed and incubated at 37 °C for 25 min and finally analyzed using the optimized LC-MRM method. To examine the effect of reaction time on the labeling efficiency, 200 μL of freshly prepared labeling reagent mixture was added into 300 μL of the same standard solution that was used to check the effect of the concentration of cyanoborohydride on the labeling efficiency. Then, the combined solution was vortexed and incubated in the UPLC sample management system, which had been set at 37 °C for 30 min before conducting this experiment. The reaction solution was analyzed using optimized UPLC-MRM method (see below) every 5 min for 60 min. For the real sample analysis, to increase the throughput, 25 μL of freshly prepared calibration standard solutions, QC samples, and rat prefrontal cortex microdialysate samples were added into different wells of a 96-well plate. Then, 10 μL of internal standard solution was added, followed by the addition of 20 μL of freshly prepared labeling reagent mixture, into each well containing standards, QCs, or rat prefrontal cortex microdialysate samples. The resulting solutions in the 96-well plate

were centrifuged, vortexed, and incubated at 37 °C for 25 min, followed by LC-MRM analyses. The labeling reagent mixture was prepared on the ice bath immediately before chemical labeling by mixing 600 μL of cyanoborohydride coupling buffer (containing 0.02 M sodium phosphate, pH 7.5, 0.2 M sodium chloride and 3.0 g/L sodium cyanoborohydride) with 200 μL of 25% acetaldehyde-d_4 in 0.75 M acetate buffer (pH 5). **Caution:** because sodium cyanoborohydride is a highly toxic chemical that will produce hydrogen cyanide gas when exposed to acid, the reductive amination was carried out in a fume hood.

Chromatography. A Waters Acquity Ultra Performance LC (UPLC) system equipped with binary solvent management and sample management with a programmable column manager (Waters, Milford, MA) was used for liquid chromatography. In brief, 20 μL of the final solution after reductive amination was injected onto a 1.7-μm particle size, 2.1 × 100 mm Acquity UPLC BEH phenyl column (Waters). The column temperature was set at 35 °C. Solvent A consisted of 0.2% formic acid and 20 mM ammonium acetate in water, and solvent B consisted of methanol. Gradient was maintained at 7% B for 0.5 min, increased from 7% B to 70% B in 2.7 min, then decreased back to the initial composition with 0.1 min, and held it for 0.2 min. The eluates were electrosprayed into an API 4000 mass spectrometer at a flow rate of 0.3 mL/min.

Mass Spectrometry. An API 4000 triple quadrupole mass spectrometer (MDS-Sciex, Toronto, Canada) controlled by the Analyst 1.4.2 software (Applied Biosystems, Foster City, CA) was used in this study. In all conditions, the following parameters remained the same: electrospray capillary voltage was 4.6 kV; source temperature was 550 °C; curtain gas was set at 35; and nebulizing, desolvation gas flow rates were set at 85. For LC/MS and LC/MS/MS analyses of derivatized and nonderivatizated NE, NM, DA, and 5-HT, declustering potential was set at 20 eV. For LC-MRM analysis of nonderivatizated and derivatized NE, NM, DA, and 5-HT, declustering potential was set at 20 and 35 V, respectively. CAD was set at 8. The resolutions of Q1 and Q3 were set at unit. The dwell time was set at 20 ms for each SRM transition.

Data Analysis. Nonderivatized and derivatized NE, DA, 5HT, NM, and their IS were detected specifically by monitoring UPLC elution times and ion pairs corresponding to the precursor and specific product ion mass/charge ratios (m/z) on a triple-quadrupole mass spectrometer operated in the positive ion SRM mode. Data acquisition and integration of the peak areas of the SRM signal for NE, DA, NM, and 5HT and their stable labeled internal standards were performed using the standard quantitative analysis package of the Analyst 1.4.2 software (Applied Biosystems). All regression analysis and sample quantification can be performed using the Analyst 1.4.2 software or the regression utility of the Watson LIMS database software. The calibration curve was constructed based on the response ratio of peak area ($P_{analyte}/P_{IS}$) versus nominal standard concentrations by either least-squares linear regression or quadratic regression using a weighting factor of either 1/concentration or 1/concentration^2 (see Table 3 for details). Concentrations of NE, DA, NM, and 5-HT were determined using the response ratio from unknown samples and the linear regression curve.

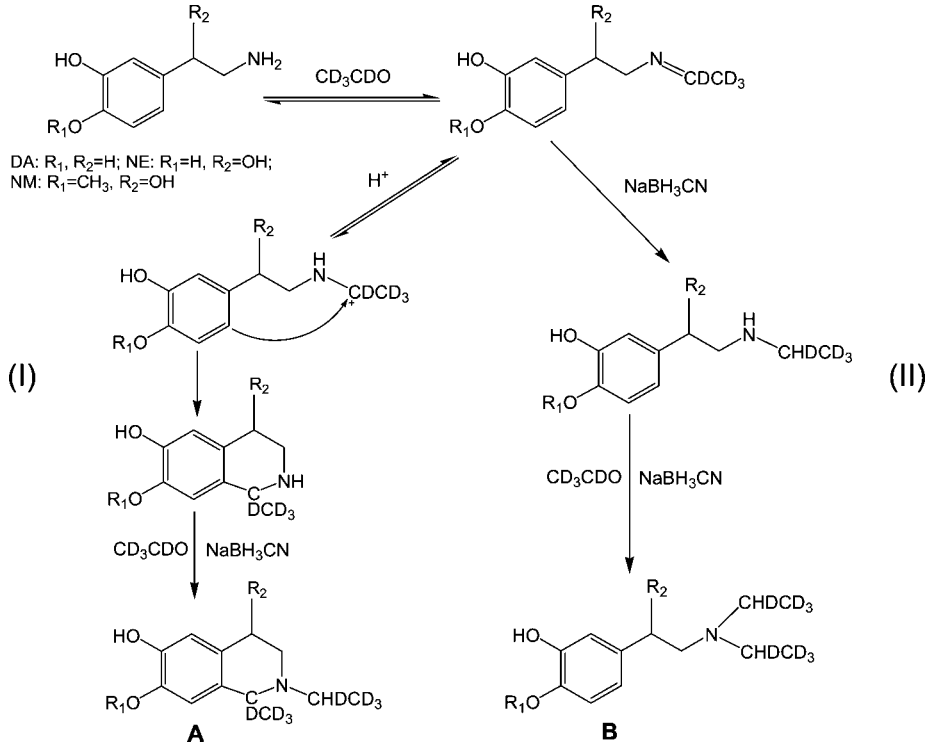
Preparation of Calibration Standards and IS. A 10 μg/mL stock solution of the NE, DA, 5-HT, and NM standards was prepared in HPLC-grade water/methanol (v/v = 80/20) in a glass volumetric flask and stored at a −20 °C freezer. To characterize the analytical performance of UPLC/MS/MS-based methods for quantitative measurement of nonderivatized and d8-diethyl-labeled monoamines, this stock solution was serially diluted using artificial CSF/preservative solution (5 μM EDTA and 100 μM citric acid) (5:1, v/v) to prepare standards at 100, 50, 25, 10, 5, 2.5, 1, 0.4, 0.2, 0.1, 0.04, 0.02, 0.01, 0.005, 0.002, and 0.001 ng/mL for each monoamine of interest. For the analysis of rat brain prefrontal cortex microdialysate samples, the stock solution was serially diluted on the day of assay using artificial CSF/preservative solution (5 μM EDTA and 100 μM citric acid) (5:1, v/v) to prepare calibration standards at 1, 0.4, 0.2, 0.1, 0.04, 0.02, and 0.01 ng/mL for each monoamine of interest. Two IS stock solutions were prepared in HPLC-grade water/methanol (v/v = 80/20): one for monoamine analysis using UPLC/MS/MS without derivatization, which contains 4 μg/mL d4-DA, 10 μg/mL d4−5-HT, and 20 μg/mL d3-NM; the other for monoamine analysis using UPLC/MS/MS with diethyl labeling, which contains 200 ng/mL d4-DA, 200 ng/mL d4−5-HT, and 40 ng/mL d3-NM. These internal standard stock solutions were diluted 100 times on the day of the assay using artificial CSF/preservative solution (5 μM EDTA and 100 μM citric acid) (5:1, v/v).

Microdialysis. MAB 4.PES microdialysis probes (200-μm o.d., 3 mm long) were equipped with hollow fiber dialysis membrane (6000 Da cutoff polyether sulfone; SciPro Inc.) and fused-silica inlet/outlet lines (180-μm o.d. made from PEEK). The microdialysis probe was implanted into the rat prefrontal cortex, a brain region innervated by dopamine neurons, over a period of 30 min to the following coordinates: 2.9 mm anterior to bregma, 0.5 mm lateral from midline, and 0.6 mm below dura. The probe was perfused with aCSF at 1.5 μL/min for at least 3 h before samples were collected for analysis. SKF-81297 (a selective D_1 dopamine receptor antagonist (1.0 mg/kg)) was prepared in 20% cyclodextrin and delivered through subcutaneous injection. Four consecutive dialysates were collected from the prefrontal cortex of a freely moving rat at an interval of 30 min to monitor basal levels of monoamines. Six consecutive dialysates were collected followed by drug administration. All brain dialysates were collected in vials containing a volume of 5 μM EDTA and 100 μM citrate equal to 20% of the intended sample volume. The typical volume of the dialysis samples was 45 μL unless otherwise noted. The dialysate samples were stored in an ice bath for immediate analysis or frozen at −80 °C for future use. Note: all experiments involving animals were carried out in compliance with national legislation and subject to local ethical review.

RESULTS AND DISCUSSION

Reductive Amination of Monoamines. Reductive amination of primary and secondary amine groups in peptides and proteins with formaldehyde has been previously reported.[17−19,25] More recently, Guo et al. has reported that there are several attractive features in using differential dimethyl labeling for relative quantification of primary and secondary amine-containing metabolites in biological samples.[24] These features include the following: (1) the experimental conditions for reductive amination are extremely mild, (2) a high yield in a relatively short

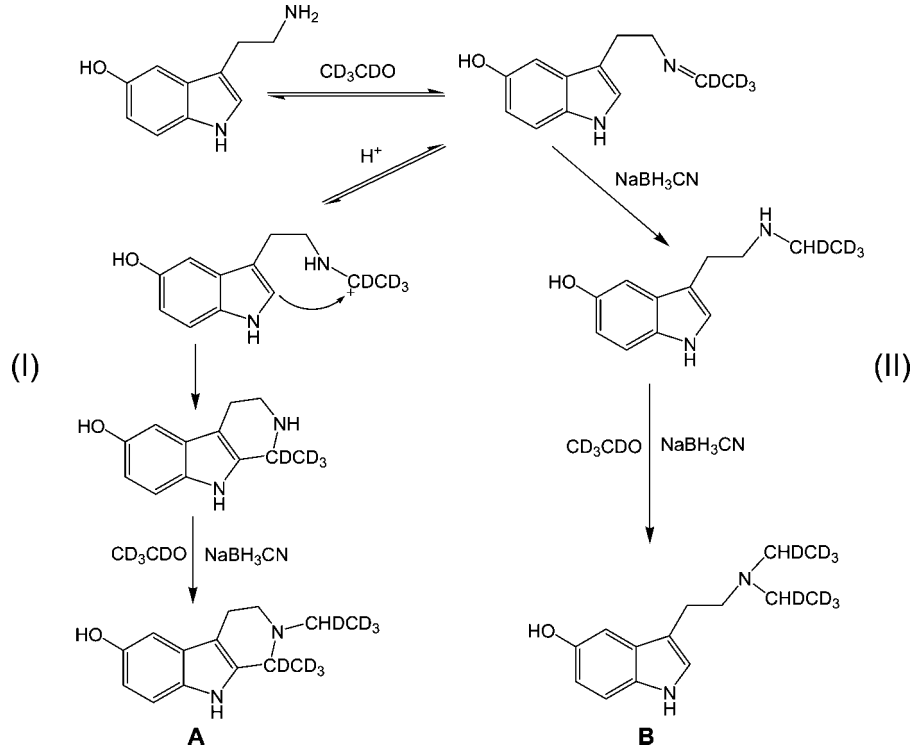
Scheme 1. Reductive Amination of DA, NE, and NM with Acetaldehyde-d_4



reaction time (10 min), and (3) the reaction is easily completed without any special reagents or reaction equipment. In this study, we extended the application of reductive amination in the enhancement of the detection limit of a conventional LC/MS/MS for simultaneously quantitative measurement of a panel of monoamines (NE, DA, NM, 5-HT) in the rat prefrontal cortex microdialysates. We investigated labeling amine groups on the monoamine neurotransmitters with formaldehyde, formaldehyde-d_2, ^{13}C-formaldehyde-d_2, ^{13}C-formaldehyde, acetaldehyde, acetaldehyde-d_3, and acetaldehyde-d_4 via reductive amination. Finally, acetaldehyde-d_4 was selected based on the following two criteria: (1) no possible interference peaks were present from the used labeling reagents or other endogenous biochemicals in microdialysis samples and (2) low-level background noise. Schemes 1 and 2 show chemical reaction process of diethyl labeling NE, DA, 5-HT, and NM with acetaldehyde-d_4. An intermediate Schiff base is formed at the initial step of this reaction process. Two main possible reaction pathways have been proposed (see Scheme 1(I),(II) and Scheme 2(I),(II)). In pathway I, the electron-donating abilities of the benzene rings attack carbon cations to form a six-member ring, which is stable, while in pathway II, the intermediate Schiff base is reduced by sodium cyanoborohydride. Then, the resulted secondary amines in pathways I and II form Schiff bases again, which are subsequently reduced by sodium cyanoborohydride to produce products A and B. Comparing the LC/MS spectra of diethyl-labeled NE, DA, NM, and 5-HT (Figure 2E−H), we can see, for NM, NE, and 5-HT, the main products are produced with the addition of 64 Da following pathway I; while, for DA, two products, following pathways I and II with the addition of 62 ($[M + H]^+ = 216.2$ in Figure 2H) and 64 Da ($[M + H]^+ = 218.1$ in Figure 2H), are almost equally formed. The possible reasons are as follows: the steric effect of the additional OH groups being added to the β-carbon of NM and NE, and the electronic accepting ability of CH_3 replaced the H of one of the phenol group making the formation of another six-member ring at the decreasing rate, DA ≫ NE ≥ NM. The electron-donating ability of 10-member ring in 5-HT is weaker than that of the 6-member ring in DA. Therefore, the main product for 5-HT derivatizated with acetaldehyde-d_4 is also B with the addition of 64 Da.

We also noted that the diethyl labeling reaction is pH dependent as described in the literature.[24] Acetate buffer (pH 5) was found to provide the optimal condition resulting in the highest yield possible for all four monoamines. Since the acetaldehyde is very volatile even at the room temperature, the pure acetaldehyde-d_4 was chilled before opening and then diluted with 1 M acetate buffer (pH 5) at the volume ratio of 1:3. The resultant acetaldehyde-d_4 concentration (~5 M) used in this study is ~100 times higher than that of sodium cyanoborohydride (47 mM) in cyanoborohydride coupling buffer. Therefore, only the effect of the reducing reagent concentration was examined and 25-min reaction time was used (see Figure 1A). We can see that the labeling efficiency for NE, 5-HT, and NM increased as the concentration of the reducing reagent increased, especially in the low-concentration range of cyanoborohydride (below 9.44 mM). The labeling efficiency for NE, 5-HT, and NM is close to the maximum when the concentration of cyanoborohydride is above 9.44 mM. However, the labeling efficiency for DA to form diethylated DA with an additional six-member ring increased as the concentration of cyanoborohydride increased in the low-concentration range (below 2.36 mM), but the labeling efficiency for DA to produce the same product reduced when continued to increase the concentration of cyanoborohydride to above 2.36 mM. The explanation for this is that the rate of reduction of the imine groups formed at the initial step of the reaction (see Scheme 1) was affected by the concentration of cyanoborohydride in the reaction mixture. At the low concentration of cyanoborohydride, the reduction of the imine groups is slow, which results in the increased chance to form a six-member ring and

Scheme 2. Reductive Amination of 5-HT with Acetaldehyde-d_4



consequently form diethylated DA with an additional six-member ring. However, after the concentration of cyanoborohydride reaches a certain point, the higher the concentration of cyanoborohydride in the reaction mixture, the higher the rate of reduction of imine groups, which results in a decreased chance to form diethylated DA with the addition of a six-member ring. To consider the labeling efficiency for all four monoamines to produce the precursor ions of interest and the dilution factor due to the volume of cyanoborohydride coupling buffer being added, in this study, 12.8 mM was chosen as the final cyanoborohydride concentration in the reaction mixture to label amino groups on the monoamine neurotransmitters. Another key element affecting labeling efficiency is the reaction time, which was also investigated in this study (see Figure 1B). We can see that the labeling reaction is almost completed for all four monoamines at the incubation time of 25 min at 37 °C using the selected reaction condition (see Experimental Section for details).

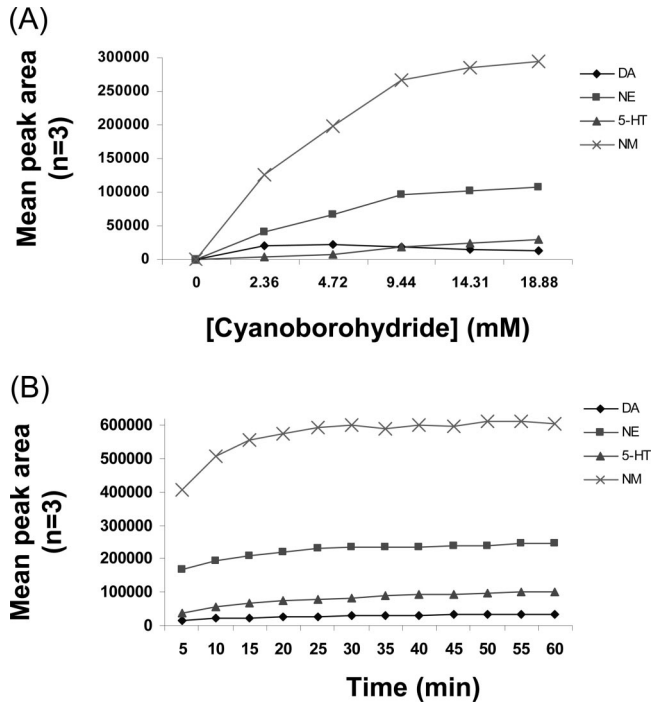


Figure 1. Effects of reducing reagent concentration (A) and reaction time (B) on reaction efficiency of diethyl labeling. The reaction was conducted at 37 °C for 25 min in (A), and 12.8 mM cyanoborohydride concentration was used in (B).

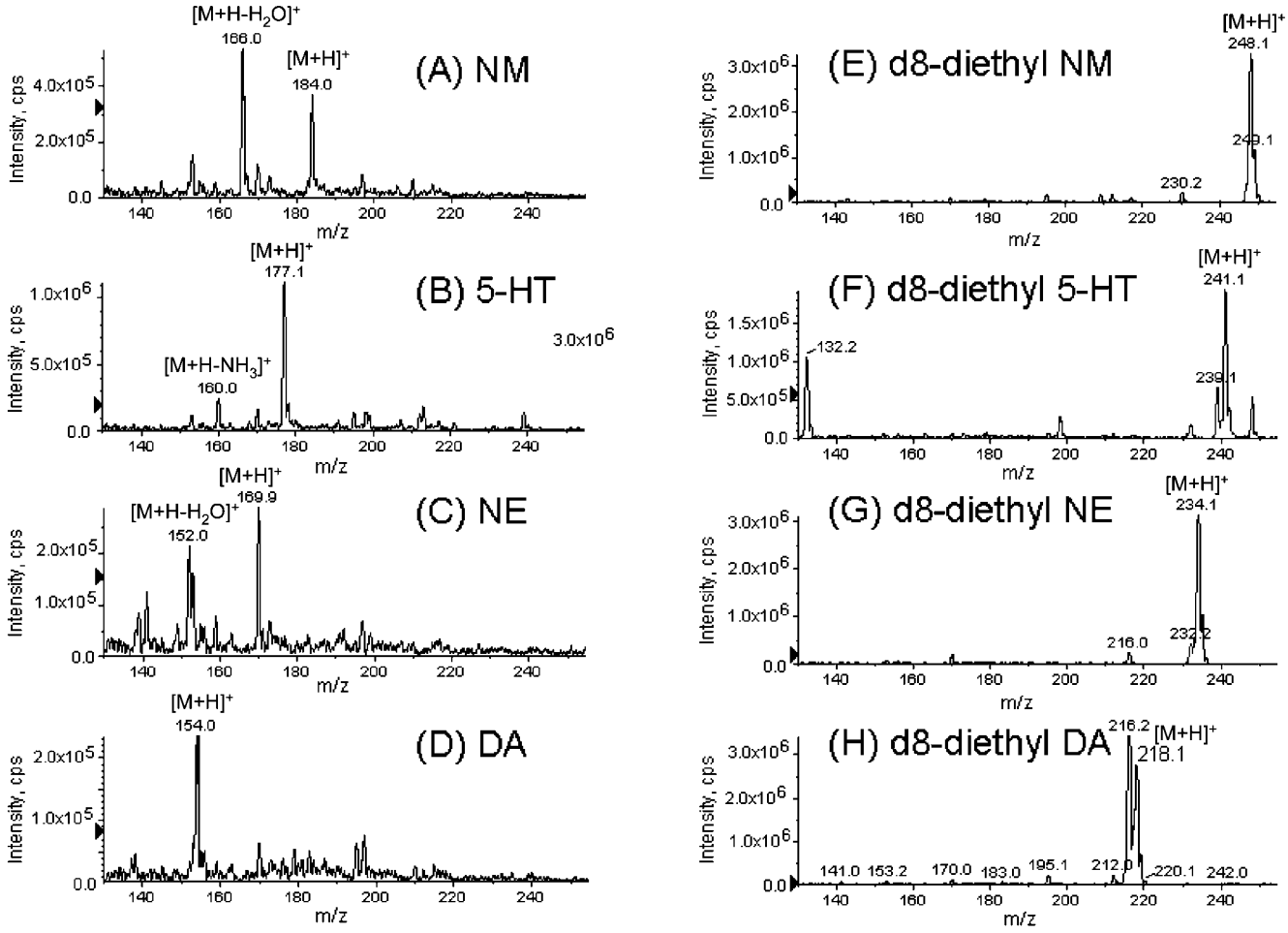


Figure 2. ESI MS spectra of nonderivatized (A) NM, (B) 5-HT, (C) DA, and (D) NE; and d8-diethyl-labeled (E) NM, (F) 5-HT, (G) DA, and (H) NE.

In summary, diethyl labeling provides a simple means of labeling amino groups on monoamine neurotransmitters, such as DA, NE, 5-HT, and NM, via reductive amination. The labeling chemistry is relatively fast (~25 min) and specific under mild reaction conditions with minimal manipulation. And the resultant diethylated products are very stable, at least within 72 h at a storage temperature of −4 °C, which was checked (data not shown).

LC ESI MS and LC ESI MS/MS Analysis of Nonderivatized and Diethyl-Labeled DA, NE, NM, and 5-HT. Figure 2 shows the mass spectra of nonderivatized and d8-diethyl-labeled DA, NE, NM, and 5-HT at the same volume and molar injection for each monoamine. By comparing the peak intensities of molecular ions of each pair of nonderivatized and d8-diethyl-labeled monoamine, we can see the peak intensities of molecular ions are increased ~6−15 times due to diethyl labeling, which resulted in increased hydrophobicity, concomitantly increased retention time on a phenyl RP column (see Table 1), and increased ionization efficiency in ESI MS analysis. A similar phenomenon has also been reported in the literature for derivatized amino acids using protonated methyl acetimidate[26] and an *N*-hydroxysuccinimide ester of *N*-alkylnicotinic acid,[16] since protonated hydrophobic analytes are more likely to migrate to the surface layer of the eletrosprayed droplets and become gas-phase ions during the solvent evaporation. In addition, the diethyl-labeled monoamines are more stable than their unlabeled counterparts, resulting in less in-source fragmentation due to the applied declustering potential (see Figure 2). This, in turn, contributes to the increased peak intensities for protonated molecular ions of diethyl labeled monoamines in the ESI MS analysis.

Table 1. Retention Time of Nonderivatized and D8-Diethyl-Labeled DA, NE, 5-HT, and NM on a Phenyl Column Using the Same Reserved Phase Gradient As Described in the Experimental Section

	retention time (min)	
monoamine	nonderivatized	d8-diethyl labeled
DA	1.25	2.17
NE	1.05	1.98
5-HT	1.95	2.66
NM	1.21	2.38

Figure 3 shows the MS/MS spectra of nonderivatized and d8-diethyl-labeled DA, NE, 5-HT, and NM. We can see that the MS/MS spectra of d8-diethyl-labeled NE, 5-HT, and NM were more simple than those that were not derivatized while the simplicity of MS/MS spectra of nonderivatized and d8-diethyl-labeled DA remains similar. This may be due to the changed fragmentation pattern resulting from diethyl labeling of monoamines. Reducing

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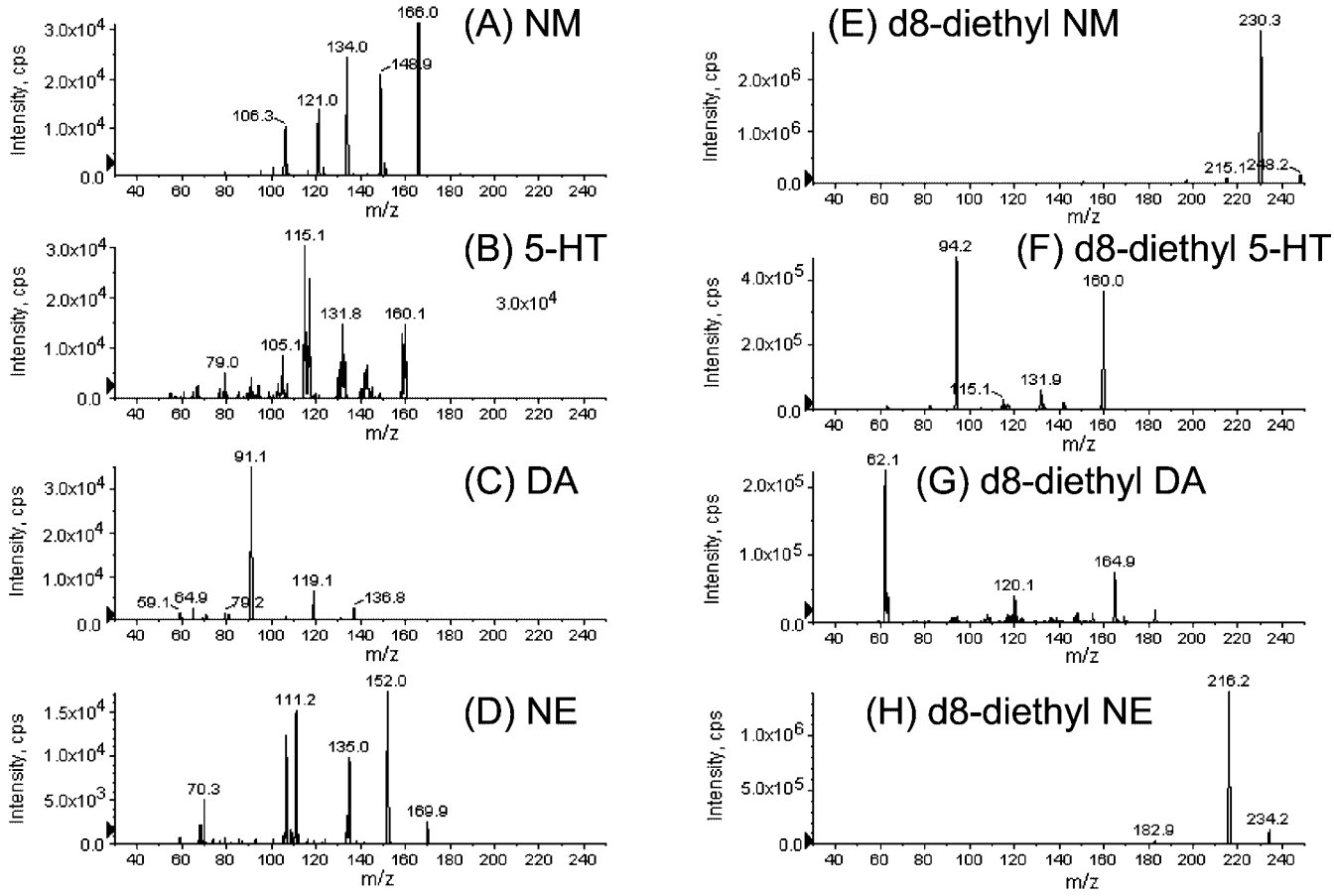

Figure 3. ESI MS/MS spectra of nonderivatized (A) NM, (B) 5-HT, (C) DA, and (D) NE; and d8-diethyl-labeled (E) NM, (F) 5-HT, (G) DA, and (H) NE.

Table 2. API 4000 Mass Spectrometry Conditions for MRM Transitions of Nonderivatized and D8-Diethyl-Labeled DA, NE, 5-HT, NM, and Their Internal Standards

	targe compound	precursor ion (*m/z*)	product ion (*m/z*)	declustering potential (V)	collision energy (eV)	collision cell exit potential (V)
nonderivatized	DA	154.1	91.1	20	36	15
	NE	170.1	107	20	29	15
	5-HT	177.1	115	20	36	15
	NM	184.1	134.1	20	28	15
	d3-NM	187.1	137.1	20	28	15
	d4−5-HT	181.1	119	20	36	15
	d4-DA	158.1	91.1	20	36	15
d8-diethyl labeled	DA	216.1	62.1	35	42	15
	NE	234.1	216.1	35	22	15
	5-HT	241.1	94.1	35	24	15
	NM	248.1	230.1	35	22	15
	d3-NM	251.1	233.1	35	22	15
	d4−5-HT	245.1	96.1	35	24	15
	d4-DA	220.1	64.1	35	42	15

the number of ions in the MS/MS spectra of diethyl-labeled monoamines could result in the increased peak intensities of the observed product ions. This could be another explanation for the increased detection sensitivity for diethyl labeled monoamines in the LC-MRM analysis (see below).

Analytical Performance of Nonderivatized and d8-Diethyl-Labeled DA, NE, NM, and 5-HT. The UPLC/MS/MS method was developed for quantitative measurement of DA, NE, 5-HT, and NM in the rat prefrontal cortex microdialysates. The detection and quantification limits of the UPLC/MS/MS-based method for nonderivatized and d8-diethyl-labeled monoamines were characterized by analyzing a series of standard mixtures, each mixture containing four monoamine neurotransmitters at different concentrations, using the optimized MRM transitions as listed in Table 2. Supporting Information Figure 1 shows the representative chromatograms of nonderivatized and d8-diethyl-labeled DA, NE, 5-HT, and NM. Table 3 lists the characterized analytical performance results of UPLC/MS/MS-based methods for nonderivatized and d8-diethyl-labeled monoamines. We can see that the sensitivity of the UPLC/MS/MS-based method for quantitative

Table 3. Analytical Performance of UPLC/MS/MS-Based Methods for Nonderivatized and d8-Diethyl-Labeled DA, NE, 5-HT. and NM

	nonderivatized	d8-diethyl labeled				
name of monoamines	limit of quantification (ng/mL)	limit of quantification (ng/mL)	dynamic range (ng/mL)	linearity	fit for progression	weighting factor
DA	0.1	0.01	0.01−1	0.9997	linear	$1/x$
NE	1	0.01	0.01−1	0.9999	linear	$1/x$
5-HT	0.2	0.005	0.01−1	0.9998	quadratic	$1/x^*x$
NM	0.4	0.002	0.01−1	0.9999	linear	$1/x$

measurement of monoamines has been increased ~20−100 times due to diethyl labeling, which results in the following: (1) increased hydrophobicity and, concomitantly, increased ionization efficiency in ESI MS/MS analysis; (2) changed fragmentation pattern. This increased sensitivity might also be due to the reduced matrix effects resulting from LC separation and reduced background noise in the MRM chromatogram because of changed transition.

Analysis of Rat Prefrontal Cortex Microdialysates. Diethyl labeling combined with UPLC/MS/MS was applied to monitor the drug-induced changes of the monoamine concentrations in rat prefrontal cortex microdialysates. Figure 4 shows the concentration changes of the monoamines upon the injection of 1 mg/kg SKF 81297. The dopamine level was gradually increased by ~3 times at 90 min after SKF 81297 was subcutaneously injected and gradually relaxed to a value still higher than the basal level of dopamine at 180 min after drug treatment. The concentration of NE, 5-HT, and NM changed with the same time course as that of DA. The similar modulation of DA and NE was observed in the rat prefrontal cortex microdialysates by using an LC-EC method after the injection of 1 mg/kg SKF 81297 (data not shown). In one of the selected rats, average basal levels of DA, NE, 5-HT, and NM (mean ± SEM) were 0.058 ± 0.011, 0.063 ± 0.008, 0.017 ± 0.001, and 0.013 ± 0.002 ng/mL, respectively, while high concentrations of the same compounds after subcutaneous injection of 1 mg/kg SKF 81297 were 0.151, 0.249, 0.075, and 0.048 ng/mL. Concentrations were not corrected for in vitro probe recovery. Representative chromatograms from the analysis are presented in Supporting Information Figure 2. All the solutes were eluted in 3.5 min. Therefore, the total chromatographic analysis time for all 10 rat prefrontal cortex mocrodialysate samples was less than 1 h (3.5 min × 10 = 35 min). The high sensitivity of this method, which could result in the requirement of small sample volume, combined with a short UPLC analysis time, will enable the consecutive analysis of the rat prefrontal cortex microdialysate samples in a high temporal resolution if it is needed.

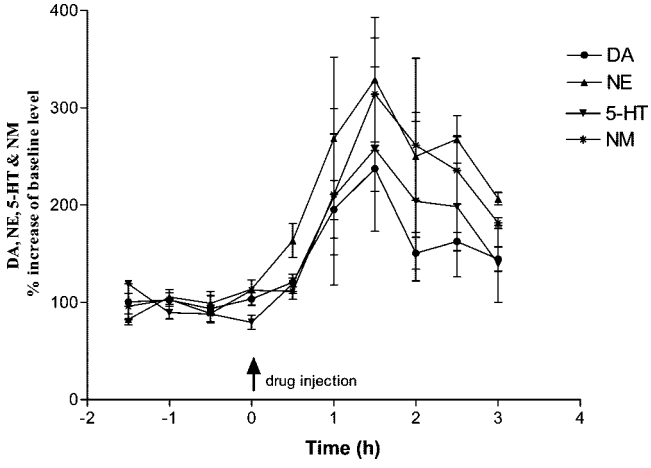

Figure 4. Concentration changes of the monoamines in the rat prefrontal cortex microdialysates after injection of SKF-81297 at the dosage of 1 mg/kg. Injection time of SKF-81297 is marked as time 0. Results were expressed as percentage increases above baseline (average of 4 untreated points, 100%). $n = 2$ rats.

CONCLUSIONS

In this work, we presented a high-sensitivity and high-throughput UPLC/MS/MS method in combination with diethyl labeling for quantitative measurement of a panel of monoamines, such as DA, NE, 5-HT, and NM, in rat prefrontal cortex microdialysates. The use of UPLC/MS/MS enabled us to separate and quantify four monoamines of interest in the rat brain microdialysate in a 3.5-min chromatographic run. To the best of our knowledge, this has been the most efficient chromatographic separation method for simultaneous measurement of DA, NE, 5-HT, and NM in the rat brain microdialysate samples. Although diethyl labeling is a shortcoming as a pretreatment step before sample injection for this UPLC/MS/MS-based method, it provides ~20−100 times increased sensitivity of a UPLC/MS/MS-based approach for quantitative measurement of DA, NE, 5-HT, and NM. Therefore, it enables the use of a UPLC/MS/MS-based method for simultaneous measurement of DA, NE, 5-HT, and NM in the rat prefrontal cortex microdialysates, in which monoamine concentrations have been reported to be very low and difficult to measure using LC/MS/MS-based approach.[12] The simple and rapid labeling reaction is carried out under very mild conditions with minimum sample and reagent manipulation. The labeling reagents are commercially available, and the labeling process is very robust and reliable. The resulting diethylated products are very stable at least within 72 h at the storage of −4 °C, which can be set for an autosampler in most of bioanalytical laboratories. Therefore, the throughput of the sample preparation could be highly improved by carrying out chemical derivatization in one or more 96-well plates. In the future, a similar practice could also be applied to analyze other primary or secondary amine-containing biochemicals in the biological matrix if high sensitivity is needed.

Compared with other reported techniques, such as HPLC-EC,[27−29] HPLC-FL,[9] and HPLC-PFET,[11] for simultaneous measurement of

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monoamine neurotransmitters in the rat brain microdialysates, diethyl labeling combined with UPLC/MS/MS provides comparable or even lower concentration detection limits. The high specificity and high throughput of this UPLC/MS/MS-based approach will enable its wide application for simultaneous measurement of monoamine neurotransmitters in the pharmaceutical industries, where monoamine neurotransmitters have been used as biomarkers in the discovery and development of new drugs to treat the disorders of central and peripheral nervous systems.

ACKNOWLEDGMENT

We thank Drs. Nalini Sadagopan, Gabriella Szekely-Klepser, and Sarah Grimwood for their support and scientific discussions. We thank Dr. Qinhua Huang for discussing and reviewing the proposed chemical process and Dr. Jiun-Tang Huang for UPLC/MS hardware and software installation and configuration. We also thank David Johnson for the discussion on using LC-EC for quantitative measurement of DA and NE in rat prefrontal cortex microdialysates and sharing the observed modulation of DA and NE in rat prefrontal cortex microdialysates after subcutaneous administration of 1 mg/kg SKF 81297. C.J. and W.L. made equal experimental contributions.

SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

Received for review June 30, 2008. Accepted September 4, 2008.

AC801339Z