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Quantification of monoamine neurotransmitters and melatonin in sea lamprey brain tissues by high performance liquid chromatography–electrospray ionization tandem mass spectrometry

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ABSTRACT

A rapid liquid chromatography–tandem mass spectrometry (LC–MS/MS) method was developed and validated for simultaneous quantification of three monoamine neurotransmitters (NTs) and melatonin in sea lamprey brain tissues. Separation was performed on a reversed-phase column with mobile phases of 1 mM perfluoroheptanoic acid (PFHA) water solution/acetonitrile and mass spectra were acquired in positive electrospray ionization multiple reaction monitoring (MRM) mode. Solid-phase extraction (SPE) was employed to purify and extract the target compounds from the tissue samples. The matrix effects as well as the influence of two extraction methods, protein precipitation (PPT) and SPE, on matrix effects were examined for the first time on the quantification of NTs from brain tissue extracts. The matrix effects with SPE (2.4 to −14.9%) were about 30% lower on average than those with the ACN PPT method (−29 to −38%). The recoveries of three types of SPE cartridges were tested and Bond-Elut C18 cartridge was selected to process the samples because of its good extraction efficiencies (71.3–95.3%) and low matrix effects (−6.6 to −14.9%) for all four analytes. This method exhibited excellent linearity for all analytes with regression coefficients higher than 0.99. The limits of detection were between 0.03 ng/mL (melatonin) and 0.14 ng/mL (norepinephrine). The precisions, expressed as coefficients of variation (CV), ranged from 4.8 to 14.1% for intra-day analyses and from 6.1 to 16.2% for inter-day analyses. Brain tissues from 360 sea lampreys were analyzed by the developed method and the concentrations for four analytes were found to be at the level of nanogram per gram of brain tissues. To our knowledge, this is the first report on the quantification of NTs and melatonin in the sea lamprey using the LC–MS/MS method.

1. Introduction

The sea lamprey (*Petromyzon marinus*) is an ancient jawless vertebrate, and a useful model in basic neurobiological research due to its crucial phylogenetic position between protochordates and jawed vertebrates (gnathostomes). This specie has a complex life cycle that includes a radical metamorphosis and adaptation to dramatic changes in its habitats. Studies using lampreys have proven useful in elucidating the ancient architecture and neural circuits of vertebrate brains [1–4]. Recently, lampreys have been used to study the evolution of vertebrate nervous system, the adaptive mechanisms of nervous system under different environments and life stages, the organization of various neurotransmitters and their functions, spinal cord regeneration, and neurotoxic effects of proteins involved in Alzheimer's disease [5].

Neurotransmitters (NTs) play an essential role in neural communication. Melatonin is a neurotransmitter-derived hormone produced primarily by the pineal gland and involved in maintaining the circadian rhythm of several biological functions. The examination of these compounds in lamprey nervous system may reveal features that are not readily discernible in the rodent and human brains. However, very few publications report quantitative analyses of NTs and melatonin in the sea lamprey brain, especially the influence of their levels on behavioral responses, environmental stimulations, and exposure of sex pheromones. One of the challenges of this study is the relative small amount of the lamprey brain tissue compared to the rat brain. The average brain weight of adult sea lamprey is less than 100 mg, while the brain weight of an adult rat is between 2 and 4 g. Therefore, it is essential to establish a sensitive and reliable method to determine NTs and melatonin in sea lamprey brain tissues for biological studies.

The separation and determination of different kinds of NTs and melatonin can be achieved by gas chromatography (GC), liquid chromatography (HLPC, UPLC), and capillary electrophoresis (CE) coupled with various detection methods including ultraviolet

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(UV) detection, fluorescence detection, electrochemical detection, laser-induced fluorescence detection, and mass spectrometry [6]. However, the applications of these methods were limited for biological research due to: a lack of reproducibility and selectivity, poor sensitivity, the necessity for an additional derivatization step, time and sample consuming, etc. [7]. Liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS), with advantages in analytical sensitivity and compound specificity, has become a powerful analytical technique for quantitative bioanalyses and has been applied to determine monoamine NTs and neuropeptides in different biological sample matrices [7–20]. Although the impact of matrix effects on the accuracy and precision of the LC–MS/MS methods is a growing concern [21–24], many published works have not addressed these issues in details. Matrix effects can be highly variable depending on the sample preparation techniques performed prior to the LC–MS/MS analyses. The differences of each sample preparation method on the matrix effects were not touched upon in the method development and validation of measuring NTs in brain tissues. Protein precipitation (PPT) with methanol or acetonitrile (ACN) is the most widely used sampling technique due to its simple and fast procedure [6,7,11–13,18–20]. However, this method often resulted in significant matrix effects because of the presence of many residual matrix components. Solid phase extraction (SPE) has been proven advantageous over other methods for removing interfering components from the matrix and pre-concentrating analytes in biological samples [8,10,14].

In this study, we present a sensitive LC–MS/MS method coupled with SPE to simultaneously quantify three monoamine NTs and melatonin in sea lamprey brain tissues with high accuracy and precision. These four analytes, dopamine (DA), norepinephrine (NE), 5-hydroxytryptamine (5-HT or serotonin), and melatonin (MLT), were quantified in the forebrain and hindbrain of sea lampreys. Sample preparation methods of PPT with ACN and SPE with three types of cartridges were tested and compared in terms of their extraction efficiencies and matrix effects. The importance of the matrix effects and the comparison of matrix effects between PPT and SPE on quantification of target analytes from brain tissue extracts were explored the first time. Brain tissues from a total of 360 sea lampreys have been analyzed by the developed method. To our knowledge, this is the first report on the quantification of NTs and melatonin in the sea lamprey with LC–MS/MS method.

2. Experimental

2.1. Chemicals

DA (purity, >98.5%), NE (purity, >98.0%), 5-HT (purity, >98.0%), MLT (purity, >99.5%), dichloromethane (HPLC grade), and PFHA (99%) were purchased from Sigma–Aldrich (St. Louis, MO, USA). The internal standard of deuterated melatonin (7D-MLT) was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). Methanol (HPLC grade), acetonitrile (HPLC grade), and formic acid (99.5%, LC/MS grade) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Distilled water was produced by a Barnstead NanoPure Infinity Ultrapure Water System (Thermo Scientific, Asheville, NC, USA). Oasis® cartridges HLB (3 mL/60 mg) were purchased from Waters (Milford, MA, USA). Bond-Elut C18 (1 mL/100 mg) and NEXUS (3 mL/60 mg) cartridges were purchased from Agilent Technologies (Santa Clara, CA, USA).

2.2. Sea lamprey brain tissue collection

A total of 360 sea lampreys were collected by the U.S. Fish and Wildlife Service (Marquette, MI) and Canadian Department of Fisheries and Oceans Sea Lamprey Center, and transported to the U.S. Geological Survey, Hammond Bay Biological Station (Millersburg, MI), where brain tissue samples were collected. For each set of experiments, all test subjects were captured from the same stream on the same day to reduce variation in levels of maturity. Standard operating procedures for transporting, maintaining, handling, anesthetizing, and euthanizing sea lampreys were approved by the Institutional Committee on Animal Use and Care of Michigan State University. All animals were anesthetized with 0.05% MS-222 (Sigma–Aldrich) before handling or tissue sampling.

Forebrain and hindbrain were separated, snap frozen in liquid nitrogen, and stored at −80 °C. The brain samples were weighed and homogenized with 400 μL of 1% formic acid in cold (−20 °C) acetonitrile [25]. One nanogram of internal standard was then added (10 μL of 0.1 ng/μL solution in 0.1% formic acid). The samples were incubated at −20 °C for 15 min and centrifuged at 15,800 × *g* for 20 min at 4 °C. The supernatant was transferred to a new tube and freeze-dried overnight.

2.3. Sample preparation

2.3.1. Protein precipitation (PPT)

The dried samples were reconstituted in 50 μL of 50% acetonitrile/water (v/v) and vortexed for 10 min. The samples were then centrifuged at 15,800 × *g* for 20 min at 4 °C. The supernatant was transferred to a new tube and stored at −20 °C until LC–MS/MS analyses.

2.3.2. Solid-phase extraction (SPE)

The dried samples were reconstituted in 0.5 mL of sodium phosphate buffer (0.01 M, pH = 7). Three types of SPE cartridges (Bond-Elut C18, Oasis HLB, and NEXUS) were tested for their extraction efficiencies of neurotransmitters in lamprey brain samples. The 12-port Supelco vacuum manifold (Sigma–Aldrich) was used for the extraction. The SPE cartridges of Bond-Elut C18 and HLB were conditioned by the successive wash of 1 mL of methanol and 1 mL of water followed by loading of 500 μL of samples. The cartridges were then rinsed with 1 mL sodium phosphate buffer (0.01 M, pH = 7) and samples were eluted with 1 mL 90% methanol/water. The Varian NEXUS cartridges were conditioned with 5 mL of 50% methanol/acetonitrile (v/v) and 1 mL of water before sample loading. The loaded NEXUS cartridges were washed with 1 mL of 50% methanol/water (v/v) and samples were eluted with 1 mL of dichloromethane. The flow rate through the cartridges was set at a rate of 3–5 mL/min. The eluted samples were evaporated using a CentriVap Cold Trap with CentriVap Concentrator (Labconco Co., Kansas, MO, USA) and reconstituted in 50 μL of 50% acetonitrile/water (v/v) for LC–MS/MS analysis.

2.4. Solution preparation

Stock solutions of the unlabeled and labeled standards were prepared by dissolving the respective compounds in 50% methanol/water (v/v) to obtain concentration of 1 mg/mL. All the stock solutions were stored at −20 °C until use. For every sample set, working solutions were prepared daily from their stock solution, and all labeled standards were analyzed by full scan MS to assure there was no detectable contamination by unlabeled analytes. Calibration solutions were prepared by spiking 10 μL of standard stock solution to brain tissue homogenates at eight concentration levels from 0.5 ng/mL to 100 ng/mL. The endogenous NTs and melatonin in the homogenates were determined and subtracted when calibration curves were constructed. The quality control (QC) samples

were prepared in homogenized brain tissue samples spiked with known amounts of analytes and internal standard at three concentration levels: 1.0 ng/mL (low, LQC), 10 ng/mL (middle, MQC), and 100 ng/mL (high, HQC).

2.5. LC–MS/MS

A Waters Quattro micro mass spectrometer coupled to a Shimadzu (Columbia, MO, USA) LC-20AD HPLC system and SIL-5000 auto-sampler was used. A Waters Symmetry C18 column (2.1 mm × 100 mm, 3.5 μm particle size) was used with column oven temperature at 30 °C. Mobile phase A consisted of 1 mM of PFHA in water, and mobile phase B was acetonitrile. The separation of 5 NTs was achieved using the following gradient program at a flow rate of 300 μL/min for 10 min: 5% B for 0.5 min; increased to 15% B from 0.5 to 2 min; increased to 25% B from 2 min to 4 min, and then maintained at 25% B from 4 to 6 min; increased to 95% B from 6 to 8 min, decreased to 5% B from 8 to 9 min and maintained at this proportion from 9 to 10 min. The injection volume was 10 μL.

Mass spectra were acquired using electrospray ionization in positive ion mode and MRM (Table 1). The capillary voltage, extractor voltage, and rf lens setting were 3.17 kV, 4 V, and 0.3, respectively. The flow rates of cone gas and desolvation gas were 20 and 400 L/h, respectively. The source temperature and desolvation temperature were 110 and 350 °C, respectively. Collision-induced dissociation employed argon as collision gas at a manifold pressure of 2×10^{-3} mbar, and collision energies and source cone potentials were optimized for each transition using Waters QuanOptimize software. This method was composed of two ESI+ functions (0–1.8 and 1.8–6.0 min) covering full run time to allow for adequate dwell time for each analyte. Data were acquired with MassLynx 4.0 and processed for calibration and for quantification of the analytes with QuanLynx software.

2.6. Method validation

Calibration linearity was studied using internal standard spiked calibration solutions at eight concentrations, ranging from the limit of quantification (LOQ) to 100 ng/mL. Each point was obtained as the average of three injections with the injection volume of 10 μL. Integrated peak areas of the selected quantification MRM transitions were used to build the curves. Curves were fitted by a weighted ($1/\chi$) least squares regression analysis using the QuanLynx function of MassLynx software. The LOD and LOQ were calculated based on the signal-to-noise ratio of 3:1 and 10:1, respectively.

Precision and accuracy were evaluated using three concentration points (LQC, MQC, and HQC). Five replicates of each point were analyzed to determine the intra- and inter-day accuracy and precision. This process was repeated three times over 3 days in order to determine the inter-day accuracy and precision using freshly prepared calibration curves. Accuracy was determined by the recovery of each QC, and precision was expressed as the coefficient of variation (CV) of the determination of QCs. Inter-day accuracy and precision were calculated similarly for the 15 replicates of each concentration point pooled from the three validation runs.

To determine the recovery, brain tissues were prepared as described above. Homogenates were pooled to obtain homogeneous sample matrices. Three samples were spiked with appropriate amount of standard stock solution before extraction. Another three samples were extracted and spiked with the same amount of standard stock solution after extraction. Three additional samples were extracted without addition of any standard solution. After correction of the spiked samples by subtracting the endogenous amounts of respective analyte, recovery was calculated by dividing the corrected mean peak area of each analyte spiked before extraction to that of each analyte spiked after extraction.

Matrix effect (ME%) was evaluated by comparing the peak area of the same concentration of analytes and IS (10 ng/mL) in standard solution of 50% acetonitrile/water (v/v) and in post-extraction of brain tissue samples. They were calculated using a modified version of the equation described by Matuszewski et al. [23]:

$$\mathrm{ME\%} = \left(\frac{\text{mean peak area in postextraction} - \text{mean peak area in blank sample}}{\text{mean peak area in standard solution}} - 1 \right) \times 100$$

The stability was investigated in the HQC (100 ng/mL)solution stored at 4 °C in the dark for 4 days from the time of sample preparation. The peak area of each compounds and IS were determined daily and compared with freshly prepared solutions.

3. Results and discussion

3.1. Mass spectrometry and chromatography

In mass spectrometric detection, positive electrospray ionization is usually used for the detection of basic compounds and negative electrospray ionization is generally preferred for acidic analytes, allowing both types of compounds to be detected as the protonated or deprotonated molecule, respectively [26]. The four studied compounds (Fig. 1), however, contain both basic (such as amino) and acidic (such as phenolic hydroxy) functional groups. Therefore, both the positive and negative electrospray ionization MRM modes were applied to test the spectrometry performance. Acquisition parameters were optimized for each compound (1 μg/mL with 10 μL injection volume) using Waters QuanOptimize software. The best response was observed in positive electrospray ionization MRM mode by monitoring the reaction m/z 170 > 152 for NE, 154 > 137 for DA, 233 > 174 for MLT, 177 > 160 for 5-HT, and 240 > 178 for IS. Table 1 shows the MRM transition and the individual cone voltage and collision energy voltages applied for the analyses.

Separation was carried out on a Waters Symmetry C18 column (2.1 mm × 100 mm, 3.5 μm particle size) within 10 min. The monoamine NTs are polar molecules poorly retained on reversed phase columns. As a result, ion-pairing reagents and gradient elution are commonly used to improve their retention [27]. The separation of melatonin can be performed on a reversed-phase column without adding ion-pairing reagents. To simultaneously separate all analytes, PFHA was used as an ion-pairing reagent to retain these polar analytes. Fig. 2 shows the chromatogram of the four analytes and IS separated in the mobile phase with PFHA. The addition of 1 mM of PFHA in mobile phase A improved the separation of all analytes with appropriate retention time and sharp peak shape. At a flow rate of 300 μL/min, the retention time was 4.89 min for NE, 5.66 min for DA, 6.04 min for MLT and IS, and 7.32 min for 5-HT.

3.2. Sample preparation methods

3.2.1. Extraction efficiencies

In this study, sample cleanup methods of both PPT with ACN and SPE with three types of cartridges were tested and compared in terms of their extraction efficiencies and matrix effects. Extraction efficiency (E%) was calculated by dividing the mean area counts of each analyte determined from the MQC (10 ng/mL) by the mean

Table 1
Mass spectrometry parameters used for analysis of NTs and melatonin.

Compound	Precursor ion (*m*/*z*)	Daughter ion (*m*/*z*)	Dwell (s)	Cone voltage (V)	Collision voltage (eV)
NE	170	152	0.1	10	10
DA	154	137	0.1	16	10
MLT	233	174	0.1	22	16
5-HT	177	160	0.1	16	10
7D-MLT	240	178	0.1	28	10

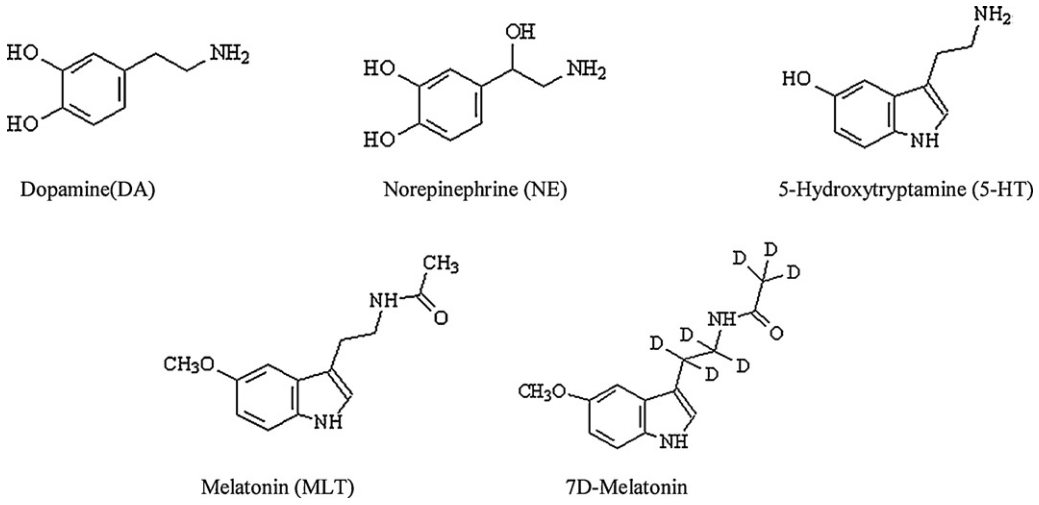

Fig. 1. Chemical structures of four studied compounds and internal standard (IS).

area counts of each analyte determined from the post-extraction spiked with the same concentration (10 ng/mL). This method eliminated the matrix effects on the calculation of *E*%, and the loss of extraction efficiencies were contributed by the extraction procedure.

Bond-Elut C18 cartridge is a single-mode silica-based reversed phase cartridge with strong hydrophobicity, while both Oasis HLB and NEXUS are mixed-mode cross-linked polymeric sorbent with a combination of hydrophilic and lipophilic moieties. The experimental conditions of Oasis HLB and Bond-Elut C18 cartridges were

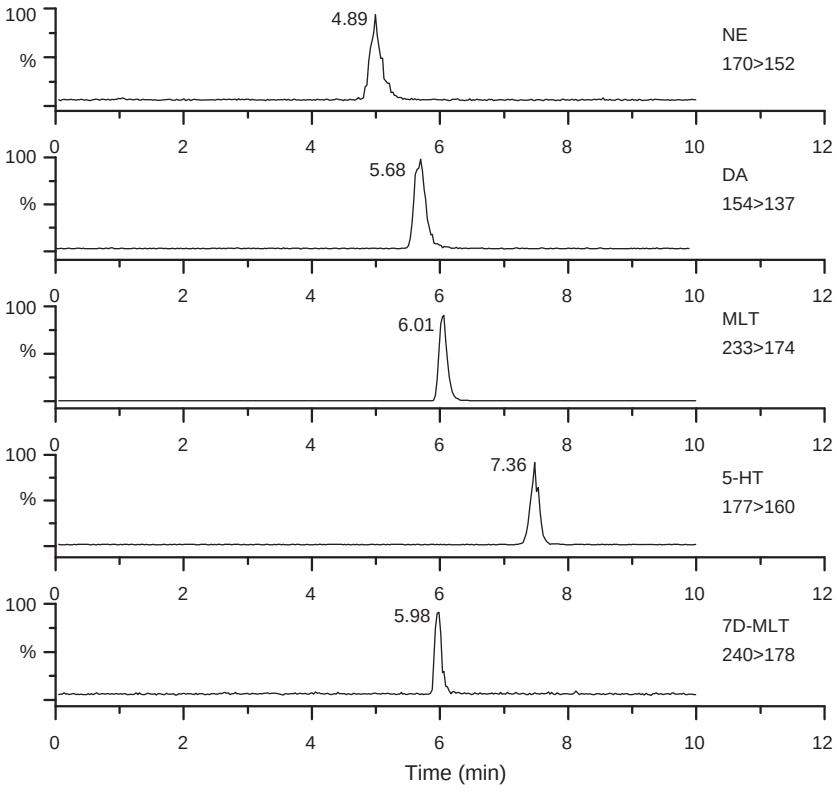

Fig. 2. Extracted LC–MS/MS chromatogram of four analytes and IS in standard solution.

the same as described in Section 2, while the extraction procedures of NEXUS followed the method reported by Eriksson et al. for the extraction of melatonin in biological samples [28]. Table 2 demonstrates the efficiencies of PPT method and SPE method using three types of cartridges for the extraction of four analytes and IS. Extraction efficiencies ranged from 82.3 to 106.1% for PPT method. All three types of SPE cartridges provided excellent efficiencies ($E\% > 91.5\%$) for MLT and 7D-MLT. The extraction efficiency of 5-HT was $(71.9 \pm 4.3)\%$ for Oasis HLB, $(86.5 \pm 2.8)\%$ for Bond-Elut C18, and $(82.5 \pm 5.2)\%$ for NEXUS cartridge. However, both Oasis HLB and NEXUS cartridge had poor recovery performance in the extraction of NE and DA.

3.2.2. Matrix effects

Matrix effect occurs when the molecules, originating from the sample matrix that co-elute with the target analytes and interfere with the ionization process during quantification by LC–MS. Residual matrix components, endogenous phospholipids in particular, are significant sources of imprecision in quantitative analyses commonly conducted by LC–MS/MS [21,22]. To determine the matrix effect, the ion response of analytes and IS spiked to the post-extraction of brain tissue samples were compared to same concentrations of analytes in neat standard solutions (50% acetonitrile/water, v/v) using the equation presented in Section 2.6. The value of ME% would indicate either ion enhancement (positive) or ion suppression (negative). Ion suppression on the analyte responses, ranging from 2.4 to 14.9% for SPE method and 29 to 38% for PPT method, was observed as shown in Table 2. When SPE was used to clean up the sample matrix, the matrix effects decreased by an average of almost 30% compared to the PPT method with ACN. Although PPT method is quick and simple, it does not result in a clean extract. Fig. 3 shows the MRM chromatograms of MLT (0.5 ng/mL) obtained in PPT post-extraction (Fig. 3A), SPE post-extraction with Bond-Elut C18 cartridge (Fig. 3B), and SPE post-extraction with HLB cartridge (Fig. 3C). Peak intensity of MLT determined in PPT post-extraction was the weakest among three sample matrices and also provided the smallest S/N ratio, which indicated strong matrix effects. The interference signals, observed from the chromatogram of the samples prepared with PPT method (Fig. 3A), revealed that interfering components can be detected in PPT post-extraction.

SPE showed advantages over PPT method by reducing the matrix effects and removing interfering components from the matrix. Although the HLB and NEXUS cartridges had poor extraction efficiencies for NE and DA, they provided cleaner extracts with small ME% (2.4–8.3%) than Bond-Elut C18 cartridges (6.6–14.9%). As displayed in Fig. 3B and C, MLT prepared in Oasis HLB post-extraction provided a stronger peak intensity and larger S/N ratio than those in Bond-Elut C18 post-extraction. These results indicated that mixed-mode SPE cartridge produced cleaner final extracts than the single mode reversed-phase SPE cartridge.

Bond-Elut C18 cartridge was chosen to process the brain samples in the following experiments because it demonstrated good extraction efficiencies and matrix effects for all four analytes. Since same degree of ion suppression was also observed for IS, the analyte/IS response ratio was unaffected during the measurement. This indicated that the quantification of NTs using the analyte/IS response ratio was independent of ion suppression, which may directly result from the matrix effect.

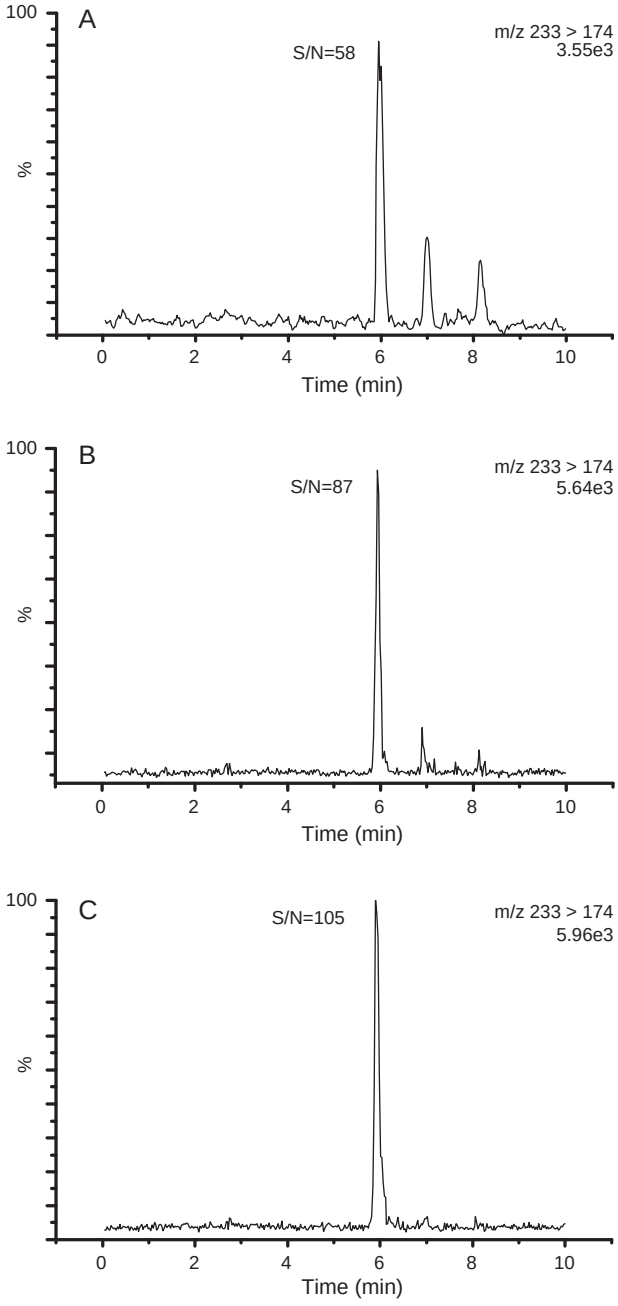

Fig. 3. Extracted chromatograms of MLT at 0.5 ng/mL analyzed by MRM. Samples were prepared by protein precipitation (PPT) (A), SPE of Bond-Elut C18 (B), and SPE of Oasis HLB (C).

3.3. Method validation

3.3.1. Linearity, limit of detection (LOD), and limits of quantitation (LOQs)

Table 3 summarizes the linearity, LOD, and LOQ of the developed method in the determination of the four analytes. Linear calibration curves were constructed in the range from 0.5 ng/mL to 100 ng/mL for each analyte using the regression of the peak area versus the concentration of the standard. No efforts were made to reach the upper concentration limits of the calibration curves. This method exhibited excellent linearity with correlation coefficients (R^2) in the range of 0.9943–0.9999. The LOQs were calculated using a signal-to-noise ratio of 10. The LODs of different analytes, which were calculated using a signal-to-noise of 3, varied from 0.03 ng/mL (MLT) to 0.14 ng/mL (NE). The LODs for NT quantifications using LC–MS/MS methods in the literature are summarized in Table 3 to compare with the method developed in this report [14,17–19,29–34]. Our method provided excellent results for the analyses of four target compounds.

Table 2
Extraction efficiencies and matrix effects of different sample preparation methods.

Compound	PPT		Bond-Elut C18		HLB		NEXUS	
	E% ± SD	ME%	*E*% ± SD	ME%	*E*% ± SD	ME%	*E*% ± SD	ME%
NE	97.6 ± 3.4	−36	71.3 ± 3.3	−7.2	34.9 ± 2.1	−5.2	13.0 ± 2.8	−3.3
DA	106.1 ± 4.5	−38	83.1 ± 4.2	−6.6	46.8 ± 3.6	−8.3	14.7 ± 3.8	−4.5
MLT	91.7 ± 6.1	−29	91.5 ± 5.6	−11.2	101.2 ± 5.4	−5.6	93.3 ± 7.6	2.4
5-HT	82.3 ± 6.6	−33	86.5 ± 2.8	−14.9	71.9 ± 4.3	−6.3	82.5 ± 5.2	−3.2
7D-MLT	89.4 ± 2.7	−36	95.3 ± 3.8	−8.5	95.6 ± 2.3	−4.5	95.0 ± 5.1	−4.0

Table 3
Analytical performance of the method.

Compounds	LDR (ng/mL)	R^2	LOD (ng/mL)	LOQ (ng/mL)	Ref. LOD (ng/mL) [Ref.]
NE	0.5–100	0.9995	0.14	0.48	0.06–1.7 [14,17–19,26,29]
DA	0.5–100	0.9998	0.09	0.29	0.03–1.5 [14,17,18,26,29–31]
MLT	0.5–100	0.9943	0.03	0.11	0.01–0.1 [32–34]
5-HT	0.5–100	0.9999	0.07	0.22	0.02–1.2 [14,17–19,26,30,32]

3.3.2. Reproducibility of retention time

The reproducibility of retention is one of the most important parameters for confident target compound identification. The method was performed over 3 days to address the reproducibility of the system. Retention times were obtained in QCs with three concentration levels. The precision of intra- and inter-day retention time (CV) ranged from 0.4 to 0.6% and 0.4 to 1.0%, respectively (Table 4). These results indicated that the HPLC conditions are reproducible from run to run.

3.3.3. Precision and accuracy

Fifteen replicates of QC samples generated at three concentrations (LQC, MQC, and HQC) from runs on three consecutive days were used to evaluate precision and accuracy at each concentration level. Table 5 summarizes the intra- and inter-day precision and accuracy for the five analytes. The intra-day precision was in the range from 4.8 to 14.1%, and the inter-day precision was between 6.1 and 16.2%. The intra-day and inter-day mean accuracies, expressed as percent recoveries, were between 75.0 and 109.8%, 69.9 and 112.1%, respectively. These results demonstrated that the values were within acceptable range, and the method was sufficiently accurate and precise.

3.3.4. Stability

Fig. 4 shows the stability of the four studied analytes and IS in HQC (100 ng/mL) solution at 4 °C in the dark for 4 days. The stability was expressed with the percentage of each analyte remained in the solution. The IS and melatonin were stable for at least 4 days under the experimental conditions, whereas 5-HT and DA were stable for up to 3 and 1 days, respectively. NE was not stable at 4 °C, and about 80% of NE was detected in the solutions after 1 day from the time of the sample preparation. The investigation of short-term stability demonstrated that NE was stable about 5 h under the experimental conditions (data not shown).

Table 4
Intra- and inter-day HPLC retention times of the five studied compounds.

Compound	Intra-day assay (n = 16)		Inter-day assay (n = 32)	
	Mean ± SD	CV (%)	Mean ± SD	CV (%)
NE	4.89 ± 0.03	0.6	4.91 ± 0.05	1.0
DA	5.66 ± 0.02	0.4	5.66 ± 0.02	0.4
MLT	6.04 ± 0.03	0.5	6.05 ± 0.05	0.8
5-HT	7.32 ± 0.04	0.5	7.33 ± 0.05	0.7

3.4. Analysis of sea lamprey brain samples

The developed method had been applied to the analysis of brain tissue samples collected from 360 sea lampreys. Fig. 5 shows the representative LC–MS/MS chromatograms of the four analytes in sea lamprey brain tissues. The net weight of collected forebrain and hindbrain tissues were in the ranges from 20.7 to 78.1 mg and 15.1 to 61.6 mg, respectively. Table 6 summarizes the mean levels of each analyte in forebrain and hindbrain tissues. The mean concentration of NE, DA and MLT in the forebrain samples was lower than those in the hindbrain samples, whereas the mean concentration of 5-HT revealed an opposite pattern. The large standard deviation of the determined concentrations was caused by many aspects such as gender, level of maturity, and treatments. For example, 5-HT concentration in the hindbrain tissue of male sea lamprey was significantly higher than that in female sea lamprey, while female forebrain tissue contained more 5-HT than males. 5-HT concentration in male forebrain was lower than the LOQ (data not shown).

There are no reported values to be compared with our results due to the lack of published research on the NT levels in sea lamprey brain. Table 6 summarizes the concentration range of three NTs and melatonin in brain of 10 fish species, including rainbow trout [35–37], telapia [38,39], carp [40], salmon [41], betta

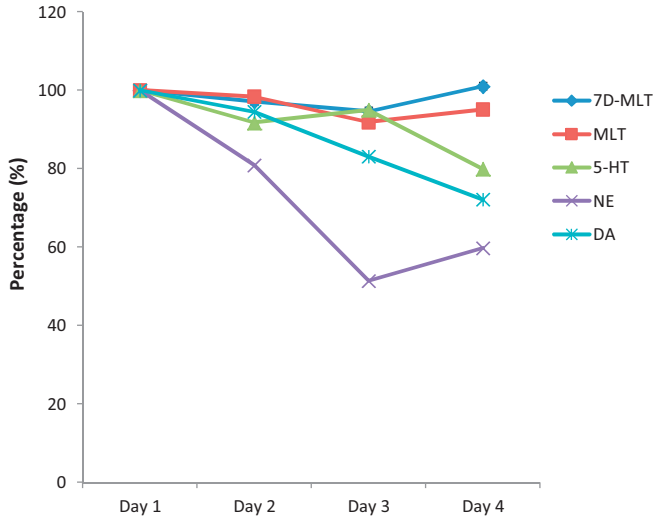

Fig. 4. Stability of three NTs and melatinin in QC solutions stored at 4 °C in dark for 4 days.

Table 5
Precision and accuracy of the method for the analysis of 5 analytes in sea lamprey plasma by SPE.

	Added (ng/mL)	Intra-day (n = 5)			Inter-day (n = 5)		
		Mean ± SD (ng/mL)	Accuracy (%)	CV (%)	Mean ± SD (ng/mL)	Accuracy (%)	CV (%)
NE	1	0.80 ± 0.08	79.8	12.2	0.86 ± 0.11	85.7	15.8
	10	7.71 ± 0.52	77.1	8.8	6.99 ± 0.51	69.9	10.4
	100	75.1 ± 3.52	75.0	6.2	70.1 ± 3.78	70.1	7.7
DA	1	1.02 ± 0.14	101.5	14.1	0.96 ± 0.14	95.7	16.2
	10	9.65 ± 0.44	96.5	4.8	9.31 ± 0.53	93.1	6.1
	100	90.7 ± 7.07	90.7	8.6	82.5 ± 6.84	82.5	10.1
MLT	1	1.03 ± 0.06	103.0	6.0	1.12 ± 0.11	112.1	9.5
	10	9.85 ± 0.49	98.5	5.1	10.1 ± 0.75	100.6	7.4
	100	91.9 ± 8.82	91.9	10.4	94.8 ± 11.5	94.8	12.7
5-HT	1	1.10 ± 0.09	109.8	7.7	0.10 ± 0.09	100.5	8.7
	10	10.1 ± 0.55	101.1	5.4	9.50 ± 0.59	95.0	6.6
	100	96.8 ± 5.61	96.8	6.0	93.3 ± 6.72	93.3	7.7

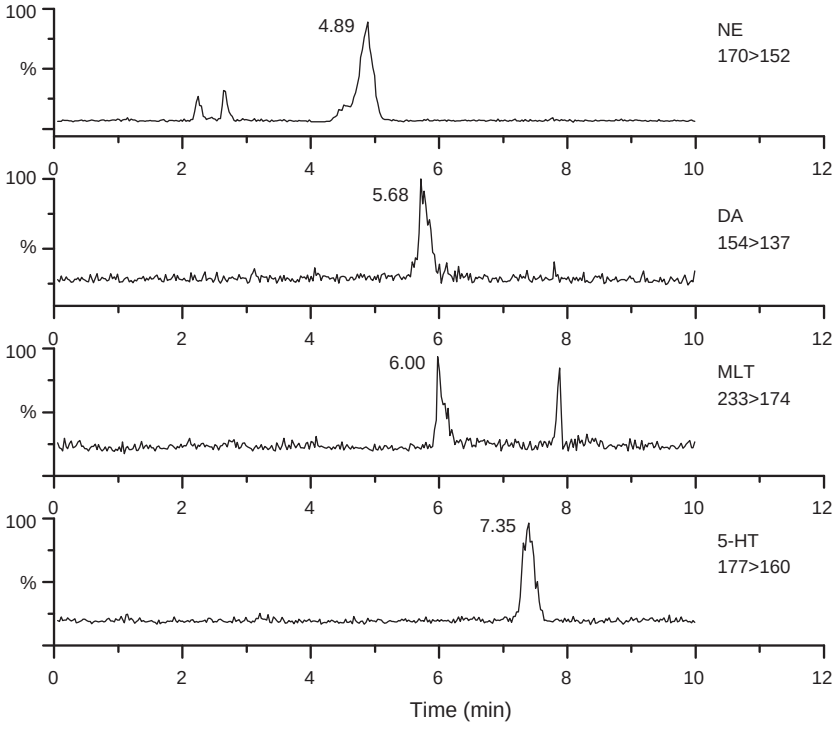

Fig. 5. Extracted chromatograms of four analytes in sea lamprey brain tissues. Samples were prepared by SPE with Bond-Elut C18 cartridges and analyzed by MRM in positive mode.

splendens [42], bass [43], fathead minnows [44], goldfish [45], and three-spined stickleback [46]. The concentrations of NE and DA determined in sea lamprey brain tissues were consistent with the results from other fish species. The MLT level in lamprey brain was higher than that in three-spined stickleback [46], whereas the 5-HT concentration was lower than other fish. Clotfelter and co-workers reported that the mean 5-HT concentration in betta splendens forebrain (220 ng/g brain tissue) was higher than that in the hindbrain (100 ng/g brain tissue) [42]. Similar trend of 5-HT concentration was observed in sea lamprey forebrain (31.9 ng/g brain tissue) and hindbrain (13.3 ng/g brain tissue) although the mean concentrations were about 10 times lower than those in betta splendens brain. Concentration levels of these compounds may vary with different fish species and are highly affected by many factors, such as the life stages, environmental stress, food intake, temperature, and the time of sample collections.

Table 6
Concentration of four analytes in the sea lamprey brain tissues.

Compound	Concentration (ng/g brain tissue) (n = 360)		Ref. concentration level in fish brain (ng/g brain tissue) [Ref.]
	Forebrain	Hindbrain	
NE	465.9 ± 240.0	954.4 ± 468.9	150–1337 [35,38,44,45]
DA	437.8 ± 248.4	599.1 ± 278.2	30–1500 [35–38,40,43,44]
MLT	0.45 ± 0.12	2.24 ± 2.04	0.05–0.025 [46]
5-HT	31.9 ± 26.8	13.3 ± 3.2	40–1045 [35,36,38–42,44]

4. Conclusions

In summary, we have developed a LC–MS/MS method for simultaneous quantification of three NTs and melatonin in sea lamprey brain tissue homogenates. Among the three types of SPE cartridges tested, the best recoveries ranging from 71.3 to 95.3% were obtained with Bond-Elut C18 cartridge. The method was validated with respect to linearity, precision and accuracy, matrix effect, and stability. Excellent linearity for all the analytes was obtained with regression correlation coefficients higher than 0.99. The limits of detection were between 0.03 ng/mL (MLT) and 0.14 ng/mL (NE). The precision results were expressed as coefficients of variation and ranged from 4.8 to 14.1% for intra-day analysis and from 6.1 to 16.2% for inter-day analysis. The intra-day and inter-day mean accuracies were between 75.0 and 109.8%, 69.9 and 112.1%, respectively. The described method is suitable for quantifying 5-HT, DA, NE and melatonin in biological samples with good reproducibility, high accuracy, as well as low intra- and inter-day variation.

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