Characterization of monoaminergic neurochemicals in the different brain regions of adult zebrafish

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Abstract

Monoaminergic neurotransmitters are the main components that regulate of a lot of processes in the vertebrate brain. There is growing interest to monitor the changes produced in these neurochemicals due to the large number of exogenous agents, such as pharmaceuticals and drugs of abuse, targeting and affecting this system. Adult zebrafish (*Danio rerio*) shares the common neurotransmitter pathways and nervous system organization with mammals. Therefore, a method based on liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) has been developed for the first time to study the profile of ten monoaminergic neurochemicals in the anterior, middle and posterior brain regions of adult zebrafish. Moreover, the applied LC-MS/MS method has been studied in terms of quality such as linearity, sensitivity and intra- and inter-day precision. The analytical method based in LC-MS/MS has become a new source in neurotoxicology using adult zebrafish as research model. Significant differences on the levels of these neurotransmitters have been found between the different brain regions.

Capsule

The profile of ten monoaminergic neurochemicals in the main three brain areas of adult zebrafish has been reported for the first time in this manuscript.

Keywords

Adult zebrafish; Brain; Neurotransmitters; Liquid chromatography; Tandem mass spectrometry.

1. Introduction

Neurotransmitters (NTs) are substances synthetized in the presynaptic neuron, that are present in the presynaptic terminals, where they are released to exert a defined action on the postsynaptic neuron or effector organ (Kandel et al., 2013). They are released into the synaptic cleft and interact with receptors from the postsynaptic neurons by regulating their membrane polarization and ionic transport (Ayano, 2017; Gómez-Canela et al., 2018; Rivetti et al., 2019). In the central nervous system (CNS), these neurochemicals are essential for neuronal communication, which is involved in basic processes such as learning, memory and behavior (Horzmann and Freeman, 2016). Monoamine NTs, specially the catecholamines dopamine (DA) and norepinephrine (NE) as well as the tryptamine serotonin (5-HT), are the main molecules that regulate a plethora of processes in the vertebrate brain, including motor control, emotion, and stress and cognition states (Maximino and Herculano, 2010). Cell bodies of their synthesizers, the monoaminergic neurons, are located in different brain nuclei and project their axons to a wide variety of specific areas (Kaslin and Panula, 2001; Maximino et al., 2016; Panula et al., 2010). There, the neurotransmitter receptors are expressed and involved in specific functions, such as motor processes, memory, mood or arousal (Panula et al., 2010). Thus, DA is synthesized from L-tyrosine whereas 5-HT from L-tryptophan (Maximino et al., 2016). Fig. SI1 (*Supplementary Information*) displays the pathways involved in the synthesis and metabolism of these NTs, which are well conserved in vertebrates (Horzmann and Freeman, 2016). Many reports have shown that exposure to different environmental pollutants, including some heavy metals, clorpyriphos, atrazine or lindane, may directly impair different components of the monoaminergic system in the fish brain (Aldegunde et al., 1999; De Boeck et al., 1995; Eddins et al., 2010; Wirbisky et al., 2015). Contaminant-induced changes in brain monoamines may link behavior and physiology in the exposed fish (Scott and Sloman, 2004), and changes in the levels of monoaminergic neurochemicals induced by pollutants have been related with altered social behavior or prey capture (Smith et al., 1995). However, the links between behavioral changes and altered monoaminergic system may be obscured by the absolute measurement of brain monoamines rather than rates of turnover and synthesis, which are likely more relevant to fish behavior (Scott and Sloman, 2004). Moreover, the analysis of the neurochemicals should be done in the specific region of the brain directly involved in the studied behavior (Gopal and Ram, 1995).

Zebrafish (*Danio rerio*) is a vertebrate model whose use in biomedical research has increased, including neurotoxicology studies, mainly due to its similarities with the organization of the human nervous system (Kalueff et al., 2014). The use of zebrafish at embryos and larvae development stages has been assessed for neuropathology processes. However, adult zebrafish is more suitable to study complicated brain disorders due to its well-developed CNS and to assess

complex behaviors (Faria et al., 2018). Interestingly, the recent development of behavioral assays in adult zebrafish has allowed the study on the psychopharmacology of monoaminergic functions in this species, finding that behavioral functions of monoaminergic neurotransmitters are highly conserved (Maximino et al., 2016). Thus, adult zebrafish should be an ideal model species to link the adverse effects of the environmental pollutants on the monoaminergic system with changes in specific behaviors which are essential for its survival in natural conditions.

Many analytical methodologies have been developed to conduct metabolomics research such as direct infusion mass spectrometry (DI-MS), high resolution mass spectrometry (HRMS), gas chromatography coupled to MS and proton nuclear magnetic resonance (^{1}H-NMR) (Asiago et al., 2010; Bajad et al., 2006; Højer-Pedersen et al., 2008). However, liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) has become a prominent technique to analyze NTs at trace levels in zebrafish (Gómez-Canela et al., 2013, 2018). Thus, an UHPLC-MS/MS method to analyze 17 NTs, precursors and neuromodulators at different developmental stages in zebrafish was performed (Santos-Fandila et al., 2015). On the other hand, NTs were also analyzed using hydrophilic interaction liquid chromatography (HILIC) coupled to MS/MS in zebrafish embryos and larvae to study how the levels are affected by their exposure to environmental pollutants (Tufi et al., 2016). Recently, a chromatographic method based on LC-MS/MS was optimized to evaluate the changes of 38 NTs in zebrafish larvae (Gómez-Canela et al., 2018), which was also subsequently applied in another study to evaluate the acute exposure to acrylamide in adult zebrafish brain (Faria et al., 2018). On the other hand, the weight of the whole brain of a 3.5-4 cm adult zebrafish is about 5 mg. However, in order to link changes in behavior with changes in the monoaminergic profile, it is important to measure the concentration of these NTs in the specific areas involved in the development of the observed behavior rather than measuring them in the whole brain. The zebrafish brain can be easily dissected in anterior, middle and posterior brain regions (Fig. SI2), being the dopaminergic and serotonergic systems mainly located in the first two regions (Kaslin and Panula, 2001; Maximino et al., 2016; Panula et al., 2010). However, the small size of some of these areas makes challenging the determination of NTs. Thus, their identification and quantification in such small size samples requires an exceptional sensitivity and selectivity in the analytical methodology. For this purpose, selected reaction monitoring (SRM) of precursor-product ion transitions complies these requirements. Moreover, the determination of individual compounds by two transitions avoids false positive identifications (Krauss et al., 2010). It also increases the instrumental detection limits (IDLs) and restricts the number of target analytes to be monitored (Gómez-Canela et al., 2013). Furthermore, the challenge to analyze these compounds due to their low concentration in complex biological tissues and high polarity can be achieved by using HILIC, which allows the targeted analytes to be separated using a mobile phase compatible

with MS (Rivetti et al., 2019). Thus, the aim of this study is to develop a new highly sensitive method using LC-MS/MS to determine the monoaminergic neurotransmitters DA, NE and 5-HT, as well as their main precursors and degradation products, in the anterior, middle and posterior brain regions of adult zebrafish.

2. Experimental

2.1. Chemicals and materials

Crystalline solid standards of serotonin hydrochloride (5-HT), 5-hydroxy-L-tryptophan (5-HTP), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanilic acid (HVA), L-tyrosine (Tyr), L-tryptophan (Trp), and dopamine hydrochloride (DA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 3-methoxytyramine hydrochloride (3-MT) was obtained from Merck (Darmstadt, Germany), 5-hydroxyindoleacetic acid (5-HIAA) was provided by Toronto Research Chemicals (TRC, Toronto, Canada), and norepinephrine (NE) was supplied by Tocris Bioscience (Ellisville, USA). All the internal labeled standards (IS) were obtained from Toronto Research Chemicals (TRC, Toronto, Canada), Merck (Darmstadt, Germany), and Sigma-Aldrich (St. Louis, MO, USA). All target compounds, molecular formulas and the main physicochemical properties are shown in Table SI1. Methanol (MeOH) and acetonitrile (ACN) HPLC grade were supplied from VWR Chemicals Prolabo (Leuven, Belgium). Ammonium formate and formic acid (FA) were supplied by Sigma-Aldrich (St. Louis, MO, USA) and Fischer Scientific (Loughborough, UK), respectively. Hydrochloric acid (HCl) 1 N and ascorbic acid were obtained from Panreac AppliChem (Darmstadt, Germany), and 2-propanol (IPA) was purchased from Scharlab, S.L. (Barcelona, Spain). Finally, ultra-pure water was obtained through Millipore Milli-Q purification system (Millipore, Bedford, MA, USA).

Stock solutions of all the unlabeled compounds were prepared at 1000 ng μL^{-1} in MeOH containing 0.1% ascorbic acid and 2.5% 1N HCl to prevent oxidation. Moreover, stock solutions of IS were dissolved at the same concentration in MeOH or ultra-pure water depending on their solubility. Once prepared in silanized amber vials (2 mL), all stock solutions were kept at -20ºC in dark to prevent degradation. Standard solutions were freshly prepared every day in starting mobile phase conditions at the desired concentration level. All standard solutions were also stored in glass vials at -20ºC. For cleanup tests, Ostro™ 96-Well Plate, 25 mg, 1/pk (Waters, Milford, MA, USA) and Oasis® WCX 1cc, 10 mg Sorbent, 30 μm Particle Size, 100/pk cartridges (Waters, Milford, MA, USA) were employed.

2.2. Fish husbandry and brain extraction

Wild type adult male zebrafish, 3.8-4.2 cm standard length, were obtained from a commercial supplier (Piscicultura Superior, Barcelona, Spain) and maintained under standard conditions in the Research and Development Centre (CID-CSIC) facilities. Animals were maintained in fish water [reverse-osmosis purified water containing 90 mg/L Instant Ocean (Aquarium Systems, Sarrebourg, France) and 0.58 mM $CaSO_4 \cdot 2H_2O$] in 2.8 L tanks (density: 8 fish/L) into an Aquaneering Zebrafish System, The main parameters of the water during fish housing were: Temperature: 28°± 1° C; pH: 6.5-7.0; Conductivity: 750-900 µS/cm; NO_3: 5-10 mg/L ; NO_2: ≈0.1 mg/L Photoperiod used was 12L:12D. Fish were fed twice a day with flake food (TetraMin, Tetra, Germany).

For sample collection, fish were euthanized by inducing hypothermic shock in ice-chilled water (2-4ºC). Brains were immediately dissected and sectioned in the standard frontal plane at two different levels, the anterior limit of the optic tectum and the posterior limit of the cerebellum (Fig. SI2). As a result of this sectioning strategy, anterior, middle and posterior brain regions were obtained (Fig. SI2) and individually stored at -80ºC for further analysis. In this study, “anterior brain region” included the olfactory bulbs, telencephalon and the preoptic area, “middle brain region” included the optic tectum, cerebellum, thalamic and diencephalic structures and locus coeruleus, and “posterior brain region” included the medulla oblongata and the initial part of the spinal cord. All procedures were validated by Animal Care and Use Committee of CID-CSIC and conducted in accordance with the institutional guidelines under a license from the local government (agreement number 9027).

2.3. Monoaminergic system extraction

In order to determine the levels of NTs in the adult zebrafish brain, different extraction strategies and cleanup procedures were tested. Herein, it is important to consider that in spite of the existing differences in NTs content among the anterior, middle and posterior brain regions the biological matrix of these areas to analyze is similar, mainly constituted by neuronal cell bodies, axons and glial cells. Therefore, to reduce as much as possible the number of animals sacrificed in this study, the anterior brain region was used in the optimization of the extraction methodology, and the middle brain region of the same animals was employed for the quality control analysis. Fig. 1 shows the summary of all extraction conditions and the cleanup procedures.

The first step of the extraction procedure was adapted from a previous study about the characterization of NTs in zebrafish larvae (Gómez-Canela et al., 2018). In all tested methods, an anterior brain region of an adult zebrafish was first spiked at 150 ng with an isotope labeled

solution (serotonin-d_4 hydrochloride (HCl), 5-hydroxy-L-tryptophan-d_4, 3,4-dihydroxyphenylacetic acid-d_5, L-tryptophan-1-^{13}C, 5-hydroxyindole-3-acetic acid-d_5, DL-norepinephrine-d_6, dopamine-1,1,2,2-d_4 HCl and 3-methoxytyramine-d_4 HCl) as internal standard (internal standard mixture, ISM, in the next sections) in order to calculate the absolute recovery to study all the methods tested. Then, 300 μL of a cold extractant solvent were added. Four different solvents were tested: ACN:H_2O (90:10), ACN:MeOH (90:10), ACN:H_2O (90:10) + 1% formic acid (FA), and ACN:MeOH (90:10) + 1% FA, which were classified as methods A1, A2, A3 and A4, respectively (see Fig. 1). After that, samples were homogenized using a bead mill homogenizer (TissueLyser LT, Quiagen, Hilden, Germany) at 50 oscillations per min during 90 sec, with three stainless steel beads (3 mm diameter). Then, samples were shaken in a vibrating plate at 4ºC for 20 min and subsequently centrifuged for 20 min at 13,000 rpm, also at 4ºC. The supernatant was filtered using 0.20 μM PTFE filters (DISMIC -13 JP, Advantec®) and kept at - 20°C until LC-MS/MS analysis. Moreover, different sample treatment strategies based in the NTs extraction followed by different cleanup steps were tested in order to remove phospholipids from samples and minimize matrix effects. First, target compounds were extracted as described before with the A3 method [300 μL ACN: H_2O (90:10) + 1% FA]. Then, two cleanup alternatives were studied: (1) Ostro™, which consists in a One Pass-Through technology based on Reverse-phase hydrophobic interaction to remove proteins, particulates, and phospholipids from samples, and (2) Oasis® WCX, a SPE sorbent whose retention mechanism is mix-mode based in ion-exchange and reversed-phase, providing superior sample preparation for strong bases and quaternary amines. In Ostro™, the mechanism consists in three steps: preparation placing the Ostro plate onto a 2 mL collection plate for processing; the sample loading and the precipitation. The extracts were loaded into individual wells of an Ostro 96-Well Plate and diluted in different proportions with cold ACN + 1% FA. In this case, three extract:solvent proportions were tested (1:3, 1:1 and 3:1), which were identified as methods B1, B2 and B3, respectively (see Fig. 1). The Ostro Plate was placed into an Ostro 96-Well Collection Plate and mounted on vacuum manifold. Aliquots were placed into chromatographic vials and were kept at -20ºC until LC-MS/MS analysis. For Oasis® WCX strategy, extracts were loaded in Oasis® WCX 10 mg cartridges and diluted one-fold with 50 mM ammonium formate. Cartridges were then washed twice (one-fold dilution) with 50 mM ammonium formate followed by ACN:IPA (50:50) and vacuum dried to remove solvents. Target compounds were eluted from the cartridges with 2 x 300 μL aliquots of 2% FA in MeOH and ACN:H_2O (85:15) + 2% FA (see Fig. 1). Finally, extracts were placed into chromatographic vials and were kept at -20ºC until LC-MS/MS analysis.

2.4. LC-MS/MS analysis

LC-MS/MS analysis was performed using an Acquity UPLC® H-Class liquid chromatograph (Waters, Milford, MA, USA) coupled to a triple quadrupole mass spectrometer equipped with an ESI source able to operate in both positive and negative ionization mode (Xevo, TQS micro, Waters, USA). In order to optimize the analysis of NTs, two different chromatographic methods were tested (methods 1 and 2). Method 1 was based in a previous study about the development of an HILIC LC-MS/MS method for the analysis of 5 monoamine NTs (Danaceau et al., 2012). Method 2 was adapted from an UHPLC-ESI-MS/MS method designed to determine 23 metabolites related to the consumption of polyphenols and their microbial metabolism (Gasperotti et al., 2014).

In method 1, an Acquity UPLC BEH Amide column (150 mm x 2.1 mm ID, particle size 1.7 μm) provided with an Acquity UPLC BEH Amide pre-column (5 mm x 2.1 mm ID, particle size 1.7 μm) (Waters, Milford, MA, USA) was employed. Column operated at a flow rate of 0.25 mL min^{-1} and 30°C. Aliquots of 10 μL of standard and/or samples were injected at 10 ± 5°C. Mobile phase consisted in milli-Q water and acetonitrile (H_2O:ACN) (95:5) containing 100 mM ammonium formate at pH 3.0 (solvent A) and milli-Q water and ACN (15:85) containing 30 mM ammonium formate at pH 3.0 (solvent B). The LC gradient started at 100 % B, decreased at 80 % B in 4 min, and held for 1 min. From 5 to 7 min, B was linearly increased to 100 %. Finally, initial conditions were re-equilibrated in 3 min, resulting in a total run time of 10 min. Desolvation gas flow was set to 900 L h^{-1}, whereas cone gas flow was performed at 150 L h^{-1}. Source temperature was set at 100°C and a capillary voltage of 2.0 kV was applied. Finally, desolvation temperature was 350°C. In method 2, an Acquity UPLC HSS T3 column (100 mm x 2.1 mm ID, particle size 1.8 μm) (Waters, Milford, MA, USA) was used. Column was set to a flow rate of 0.40 mL min^{-1} at 40°C. Aliquots of 10 μL of standard and/or samples were injected at 10 ± 5°C. Mobile phase consisted in milli-Q water with 0.1% FA (solvent A) and ACN with 0.1% FA (solvent B). The LC gradient started at 5 % B, increased to 20 % B in 3 min, and held for 1.5 min. From 4.5 to 9 min, B linearly increased to 45 %, then increased to 100 % B from 9 to 11 min, and held for 2 min. Finally, initial conditions were attained in 2 min, and were then re-equilibrated in 2 min, resulting in a total run time of 17 min. Herein, desolvation gas flow was set to 800 L h^{-1}, whereas cone gas flow was performed at 50 L h^{-1}. Source temperature was set at 150°C and a capillary voltage of 3.5 kV was applied. Finally, desolvation temperature was 500°C. In both methods, NTs were measured under positive electrospray ionization (ESI+), using N_2 as desolvation and cone gas. Flow injection analysis (FIA) was employed to obtain the optimum cone voltage (C.V.) and collision energies (C.E.) of each analyte, which were adapted from previous studies (Danaceau et al., 2012; Gasperotti et al., 2014), allowing the determination of the precursor ion and at least two intense

fragments for each compound. The optimized C.V. of each compound was employed to obtain the precursor ions. They were subsequently fragmented to produce at least two intense product ions after the application of the optimized C.E.. The first transition, which corresponded to the most intense fragment, was used as the quantifier ion, whereas the second as the qualifier ion. Hence, acquisition was performed in SRM mode. Final transitions and optimized mass parameters are shown in Table 1. Internal and external quantification was used. MassLynx® Software v 4.1 (Waters, Manchester, UK) was the software used to acquire and process the data.

2.5. Quality assurance

Milli-Q water and ACN (15:85) containing 30 mM ammonium formate at pH 3.0 were used to prepare the calibration standards. Linearity was performed over a concentration range from 0.005 to 5 ng μL^{-1}, using at least six calibration points. ISM was used as internal labeled standards for extraction and analytical quality control. The target compounds 5-HT, DA, 5-HTP, 3-MT, DOPAC, Trp, 5-HIAA and NE were quantified by internal calibration, whereas the remaining 2 neurotransmitters (Tyr and HVA) were determined using external calibration. The validation of the method was checked with the recovery studies with seven replicates. For each replicate, one zebrafish brain was spiked at 150 ng with the neurotransmitter standard mixture and the ISM. In addition, the matrix effect (ME) was calculated following a previous published paper about the therapeutic potential of N-acetylcysteine in acrylamide acute neurotoxicity in adult zebrafish with minor modifications (Faria et al., 2019) using the same standard solution than in the calibration step. Thus, ME was assessed by comparing the peak area of analyte from the spiked zebrafish brain with the peak area of the analyte from the standard solution at the same concentration in mobile phase solvent (N = 4), following the equation:

$$ME\ (\%) = \frac{A - B}{C}\ x\ 100$$

where A is the peak area of each analyte from spiked zebrafish brain samples; B is the peak area of each analyte from non-spiked zebrafish brain; and C is the peak area of each analyte in mobile phase. . Instrumental detection limits (IDLs) were determined using the lowest concentration standard solution at 0.005 ng μL^{-1} (except for Trp at 0.010 ng μL^{-1} and HVA at 0.050 ng μL^{-1}) that yielded a S/N ratio equal to 3. Method detection limits (MDLs) were calculated using zebrafish middle brain region samples spiked at 150 ng that also produced a S/N ratio equal to 3. Moreover, method quantification limit (MQL) was calculated in the same way but with a S/N equal to 10. Intra-

day precision was assessed by four consecutive injections of 1 ng μL^{-1} standard solution, and inter-day precision was determined by measuring the same standard solution on four different days. The analysis of solvent blanks showed no carryover effect during the LC-MS/MS analysis.

2.6. Data analysis

Levels of neurochemicals in the three main brain areas of adult zebrafish control samples were normalized per (1) brain region and (2) weight of each brain region. One-way ANOVA followed by Tukey's multiple-comparison tests ($p < 0.05$) were applied to compare the level differences of each neurochemical among brain regions.

To perform the statistical analyses, NTs concentrations between MDL and MQL were used unaltered for calculations (Joerss et al., 2019), whereas values below MDL were treated as MDL normalized per square root of two.

3. Results and Discussion

3.1. Ionization parameters and mass spectral characterization

The 10 monoaminergic neurochemicals were identified at the optimal working conditions. Their mass spectral characterization has been previously reported in different studies (Gasperotti et al., 2014; Gómez-Canela et al., 2018, 2019) (see Table 1), some of them using high resolution mass spectrometry (HRMS). The two transitions obtained were chosen to acquire in SRM mode to obtain selectivity and sensitivity. The optimum cone voltages ranged from 14 to 56 V generating the protonated molecule $[M+H]^+$ in all cases, except for 5-HT and DA that presented the loss of an ammonia molecule $[M\text{-}NH_3]^+$ as base peak. Then, CE were optimized from 4 to 35 eV. Table 1 shows the optimum CV and CE for each compound. Two transitions were optimized choosing the precursor ion following the Directive 2002/657/CE recommendations (European Parliament and the Council of the European Union, 2002).

3.2. Optimization of the extraction procedure

Method performance was tested using seven replicates of the anterior brain region spiked at 150 ng of an unlabeled neurotransmitters mix and the respective ISM at the same concentration. Table SI2 reports all recovery results in all tested methods. Sample extraction followed by a cleanup using Oasis® WCX cartridges provided poor results, with seven non-recovered neurochemicals. However, the cleanup strategy using Ostro™ amended the results (methods B1,

B2 and B3). Method B1 provided good results recovering 7 NTs (70±12% to 153±20%), method B2 recovered 9 NTs in the range of 18±3% to 167±26% and method B3 achieved to recover 9 NTs with levels between 23±3% and 90±5% except for NE, which registered a very poor result (7±0.1%). The recovery values obtained in both cleanup procedures were due to their retention mechanisms, which kept neurochemicals as well as phospholipids and proteins from samples. Despite the improvement observed using Ostro™, the extraction of neurochemicals without a cleanup step offered the best results. Moreover, in method A2, whose extractant solvent did not contain FA, 5-HIAA was not recovered, suggesting that FA contributed to optimize its extraction. Thus, the best results were obtained in method A3, recovering all the analytes in the range of 69±17% to 133±20%. Hence, monoamine NTs were extracted from samples with ACN:H_2O (90:10) + 1% FA as extractant solvent using the extraction procedure detailed in the previous section 2.3.

3.3. Optimization of UPLC conditions

To optimize the analysis of neurotransmitters, two different chromatographic methods were tested to increase the instrumental response and to get better chromatographic parameters (such as retention of the analytes and peak resolution). In method 1, an Acquity UPLC BEH Amide column (150 mm x 2.1 mm ID, particle size 1.7 μm) provided with an Acquity UPLC BEH Amide pre-column (5 mm x 2.1 mm ID, particle size 1.7 μm) was tested, whereas an Acquity UPLC HSS T3 column (100 mm x 2.1 mm ID, particle size 1.8 μm) was used in method 2. Details about the flow rates, mobile phases and % of solvents A and B in each method are indicated in the previous section 2.4. In order to check the suitability of the chromatographic method, a 2 ng μL^{-1} standard solution containing a mix of all NTs was injected using methods 1 and 2. The multiple reaction monitoring (MRM) chromatograms obtained in both methods are shown in Fig. SI3. In method 1, a good selectivity was obtained as all the analytes were well retained in the range of 1.68 to 3.95 min. Moreover, it allowed to determine all the analytes in a relatively short retention time (< 5 min). On the other hand, a poor retention was observed in method 2 as all the analytes were eluted between 0.6 to 0.7 min except 5-HIAA, which was retained at 3.48 min. Furthermore, better peak shape and peak resolution were registered in method 1 than in method 2, according to Fig. SI3. Finally, sensitivity was evaluated by determining the IDLs at 2 ng μL^{-1} as described in the previous section 2.5. Results indicated a better sensitivity in method 1 (see Table SI3), as an increase in the S/N ratio was observed in all the compounds except in DA. Hence, the better chromatographic parameters obtained in method 1 led to select the Acquity UPLC BEH Amide column (150 mm x 2.1 mm ID, particle size 1.7 μm) with the chromatographic conditions abovementioned to its further

development and validation. HILIC describe a chromatographic technique where the analytes interact with a hydrophilic stationary phase and are eluted with a relatively hydrophobic binary eluent in which water is the stronger eluting member (Gómez-Canela et al., 2018). HILIC has been steadily gaining interest and in the last few years it has emerged as a viable option to reverse phase (RP)-HPLC for many applications dealing with polar and hydrophilic analytes. The high polarity of target compounds indicated as log P in Table SI1 make that HILIC column be the best option for the analysis of monoaminergic NTs in brain of zebrafish. The MRM chromatograms of the 2 ng μL^{-1} standard solution containing a mix of all monoamine NTs are shown in Fig. 2.

3.4. Quality parameters

Table 2 shows the quality parameters obtained by UPLC-MS/MS. External calibration was used to quantify Tyr and HVA, whereas the remaining compounds were determined by internal calibration. All of them corrected MS responses and ensured an exact quantification. Good correlation coefficients (r^2) were over 0.99 for all analytes in a range from 0.005 to 2.5 ng μL^{-1} in most cases, except for Trp (0.010 to 1 ng μL^{-1}) and HVA (0.050 to 5 ng μL^{-1}). Moreover, IDLs ranged from 0.53 pg (3-MT) to 49.8 pg (HVA), whereas MDLs varied from 0.17 to 10.0 ng middle $brain^{-1}$ and MQLs from 0.58 to 33.5 ng middle $brain^{-1}$ Furthermore, intra-day precision ranged from 0.1% to 6.0% and inter-day precision values were from 4.9% to 15.5% at 1 ng μL^{-1}. Matrix effect (ME), which may cause ionization suppression or enhancement of the analytes, was determined in samples. ME was calculated by comparing the response of spiked adult zebrafish middle brain region with a [milli-Q water and ACN (15:85) containing 30 mM ammonium formate at pH 3.0] spiked extract. Compounds with values below 100% indicated signal suppression due to the matrix, whereas values above 100% suggested a signal enhancement. No ME was observed (70%-130%) for all the analytes, as can also be seen in Table 2.

3.5. Study of the neurotransmitters profile in zebrafish brain regions

The developed method was then used to measure the levels of ten NTs, including 5-HT, DA and NE as well as some of their precursors (Trp, Tyr and 5-HTP) and the main degradation products (5-HIAA, DOPAC, HVA and 3-MT) in the three selected regions of the adult zebrafish brain. Levels of six of the NTs were above the MQL in all the analyzed samples (Tables SI4a, b). 5-HIAA exhibited levels between MDL and MQL in the 44%, 11% and 44% of the samples from the anterior, middle and posterior brain regions, respectively. Moreover, 5-HTP exhibited levels below MDL in the 44%, 22% and 67% of the samples from the anterior, middle and posterior brain

regions, respectively. DOPAC levels were between MDL and MQL in only one sample from anterior brain and other sample from the posterior brain. Finally, Trp levels were above MDL in all samples, except in one anterior brain region sample.

When the effect of the brain area on the content of the catecholaminergic neurochemicals was tested, a significant effect was found for DA ($F_{2,24}$=4.7, P=0.020) and NE ($F_{2,24}$=4.0, P=0.033). Fig. 3 shows that the levels of these two neurochemicals in the middle region were significantly higher than in anterior region (P=0.016 and P=0.036 for DA and NE, respectively; one-way ANOVA with Tukey's multiple-comparison test). The effect of the brain regions on the content of serotonergic neurochemicals was also tested, finding a significant effect for the precursor Trp ($F_{2,24}$=5.7, P=0.010), 5-HT ($F_{2,24}$=5.8, P=0.009) and its degradation product 5-HIAA ($F_{2,24}$=7.0, P=0.004). Fig. 3 shows that, as the catecholaminergic neurochemicals tendency, the levels of these three serotonergic neurochemicals were significantly higher in the middle than in the anterior brain region (P=0.008, P=0.008 and P=0.007 for Trp, 5-HT and 5-HIAA, respectively; one-way ANOVA with Tukey's multiple-comparison test). These results provide the first report of the monoaminergic NTs levels in the three brain regions of adult zebrafish.

Whereas the above results provide information about the total content of 10 monoaminergic neurochemicals in the three brain regions, their direct comparison is difficult because the size of each region is different. Therefore, the weight of the three brain regions was measured in a group of adult zebrafish with a standard length similar to those used for the neurochemical determination. The weight (mean ± standard error) for anterior, middle and posterior brain was 0.96±0.05, 3.37±0.13 and 1.32±0.11 mg, respectively. Thus, the weight of middle and posterior brain is 3.5 and 1.4 times higher than the anterior brain weight, respectively. When the content of neurochemicals in each brain region was analyzed using the mean weight values for normalization, the NTs profile was completely different (Fig. 4). When the effect of the brain region on the content of the catecholaminergic neurochemicals was tested, a significant effect was found for DA ($F_{2,24}$=911, P=0.000), the degradation products DOPAC ($F_{2,24}$=15.5, P=0.000), HVA ($F_{2,24}$=5.1, P=0.015) and 3-MT ($F_{2,24}$=80.1, P=0.000), and NE ($F_{2,24}$=48.3, P=0.000). Moreover, the levels of these NTs were higher in anterior than in posterior brain, followed by middle brain (Fig. SI4). Then, the effect of the brain region on the content of the serotonergic neurochemicals was also tested, finding a significant effect for the precursor Trp ($F_{2,24}$=3.6, P=0.042), 5-HTP ($F_{2,24}$=7.3, P=0.003), 5-HT ($F_{2,24}$=1177, P=0.000) and its degradation product 5-HIAA ($F_{2,24}$=6.3, P=0.006). The tendency of the levels of 5-HT was similar to the catecholaminergic neurochemicals (anterior brain region>posterior brain region>middle brain region. However, no differences were found between middle and posterior brain in 5-HTP and 5-HIAA levels (Fig. SI4).

Given the large number of pharmaceutical agents and drugs of abuse targeting the monoaminergic system and their tendency to affect these NTs, there is a need to develop accurate methods that allow a sensitive and simultaneous determination of monoamines, as well as their precursors and metabolites, within specific brain regions known to send or receive monoaminergic inputs (Allen et al., 2017). In rodents, different analytical methods are currently available to determine monoaminergic neurochemicals in discrete brain regions (Allen et al., 2017; Sørensen and Johannsen, 2019; Tschirner et al., 2016). Although zebrafish is currently a vertebrate model increasingly used for neuropharmacology research and studying complex brain disorders (Kalueff et al., 2014), the few available methods to determine monoaminergic neurochemicals in adult zebrafish have only been validated in the whole brain (Chatterjee and Gerlai, 2009; Faria et al., 2018; Wirbisky et al., 2015). As monoaminergic circuits in the zebrafish brain are neuroanatomically restricted (reviewed by (Panula et al., 2010)), local changes in the content of neurochemicals could be diluted in the whole brain context. Thus, analytical methods suitable to determine the neurochemical profile in specific regions of the zebrafish brain, with a very small size (≈ 0.9 - 3.5 mg), are urgently needed in the aquatic toxicology field. To our knowledge, there is only one report determining the monoamine 5-HT in the tree main areas of the adult zebrafish brain, forebrain, midbrain and hindbrain (Maximino et al., 2013). In that study, strong differences were found in the 5-HT content in response to geotaxis and scototaxis, emphasizing the importance of analyzing the NTs content in each brain area. However, a clean dissection of the forebrain (the diencephalon and the telencephalon), the midbrain (the roof of the midbrain, or tectum often referred to as the optic lobe, or optic tectum and the tegmentum) and the hindbrain (the myelencephalon, or medulla oblongata, and the metencephalon, which consists of the pons and cerebellum), on a fresh zebrafish brain and in a time short enough to avoid degradation of neurochemicals is an unrealistic option for most of the labs. However, the proposed sectioning of the brain in the anterior, middle and posterior region, using as anatomical references the anterior limit of the optic tectum and the posterior limit of the cerebellum (Fig SI2), is extremely easy, fast and available for any zebrafish laboratory. Anterior and middle brain will be the regions of interest for studying changes in the dopaminergic system, whereas the middle brain is the most suitable region for studying changes in the serotonergic system (Kaslin and Panula, 2001; Maximino et al., 2016; Panula et al., 2010).

The process to determine the wet weight of the different regions of the zebrafish brain is time-consuming, and during this time in which samples are frozen some neurochemicals may be degraded. Whereas it is convenient to normalize the data by weight to compare neurochemical levels between the different brain regions, we can envisage that for most of the applications of the proposed analytical method in the field of environmental toxicology, weight normalization will not

be necessary. Thus, one of the most evident applications of this methodology is in environmental toxicology, to assess the neurotoxic effect of pollutants on the monoaminergic profile in the anterior or middle brain regions. In these studies, each neurochemical is compared in the same brain region between control fish and treated-fish of a similar size, and weight normalization is not needed.

Whereas the suitability of the developed analytical method to determine the content of monoaminergic neurochemicals in the main brain regions has been demonstrated, the main value of this methodology in the field of aquatic toxicology is the possibility of linking behavioral and physiological changes in fish exposed to neurotoxic compounds. The analytical methodology presented here should be a powerful new tool for determining changes in monoaminergic neurochemicals in specific brain areas of fish directly involved in the studied behaviors. Thus, in order to directly compare the monoaminergic profile in a specific brain region between control and contaminant-exposed fish, it is not only unnecessary to obtain the fresh weight of the selected region during the sampling step, but could also result in the degradation of some of the targeted neurochemicals.

4. Conclusions

A method based in LC-MS/MS has been developed to determine the levels of ten monoamine neurotransmitters, including serotonin, dopamine and norepinephrine, as well as some of their precursors (L-tryptophan, L-tyrosine and 5-hydroxy-L-tryptophan) and the main degradation products (5-hydroxyindoleacetic acid, 3,4-dihydroxyphenylacetic acid, homovanilic acid and 3-methoxytyramine) in three brain regions of the adult zebrafish (anterior, middle and posterior). The applied method was studied in terms of quality such as linearity, reproducibility and sensibility. Moreover, an extraction protocol was optimized to remove phospholipids and proteins from samples and was evaluated according to the recovery values of the targeted analytes, which provided good results. Besides, no matrix effect was observed in all neurotransmitters, as all results ranged from 70 to 130%. This analytical method is suitable for the analysis of ten compounds in a single injection, allowing the compounds to be unequivocally identified with an excellent sensitivity using tandem mass spectrometry.

In this manuscript, the profile of 10 monoaminergic neurochemicals has been reported, for the first time, in the three main areas of the adult zebrafish brain (anterior, middle and posterior brain regions). Results reported significant differences on the content of each neurotransmitter between the three selected brain regions. These results have been reported for the first time in this study, and represent the basis of knowledge about the neurotoxicology of the monoaminergic functions. One of the main applications of the methodology reported here in aquatic toxicology is the possibility of linking behavioral changes with changes in the profile of the different neurochemicals in selected regions of the brain after exposure of fish to different environmental pollutants.

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Table 1. MS/MS parameters for neurotransmitters. CV: cone voltage; Collision energies (CE) for quantifier and qualifier ions are given in parentheses.

Monoamine neurotransmitter	**CV (V)**	**Parent ion (*m/z*)**	**Quantifier (*m/z*)**	**CE (eV)**	**Qualifier (*m/z*)**	**CE (eV)**
Serotonin	56	160	132	14	115	24
Serotonin-d_4 HCl	28	181	164	8	136	21
5-hydroxyindoleacetic acid	34	192	146	14	91	36
5-hydroxyindole-3-acetic acid-d_5	34	197	151	8	95	34
5-hydyoxy-L-tryptophan	36	221	204	8	146	32
5-hydroxy-L-tryptophan-d_4	36	225	207	8	137	26
L-tryptophan	14	205	188	7	146	14
L-tryptophan-1-^{13}C	14	206	189	10	146	16
Dopamine	14	137	119	18	91	20
Dopamine-1,1,2,2-d_4 HCl	14	158	141	12	123	12
L-tyrosine	24	182	165	9	136	14
3,4-dihydroxyphenylacetic acid	18	168	168	4	77	26
3,4-dihydroxyphenylacetic acid-d_5	30	174	174	4	115	28
Homovanilic acid	14	183	137	14	122	26
3-methoxytyramine	22	168	151	18	91	24
3-methoxytyramine-d_4 HCl	26	172	155	10	94	24
Norepinephrine	14	152	135	14	107	14
DL-norepinephrine-d_6 HCl	18	176	158	6	139	14

Table 2. Quality parameters obtained by LC-MS/MS for the 10 monoaminergic neurochemicals. F: slope, r^2: regression coefficient; IDL: instrumental detection limit; %R: recovery; RSD: relative standard deviation; %ME: matrix effect; MDL: method detection limit.

Monoamine neurochemicals	Linearity (ng μL^{-1})	Calibration type	F	r^2	IDL (pg)	Intra-day precision (RSD, %)	Inter-day precision (RSD, %)	%R ± RSD	%ME ± RSD	MDL (ng middle $brain^{-1}$)	MQL (ng middle $brain^{-1}$)
5-HT	0.005-2.5	Internal	0.51	0.9987	0.61	0.8	4.9	108±7	115±8	1.3	4.3
5-HIAA	0.005-2.5	Internal	5.78	0.9997	6.4	1.9	8.2	91±17	103±4	0.19	0.63
5-HTP	0.005-2.5	Internal	1.67	0.9940	35.6	0.6	7.8	102±2	105±3	0.09	0.2
Trp	0.010-1	Internal	0.99	0.9955	1.2	0.4	10.3	120±8	113±8	2.19	7.29
DA	0.005-2.5	Internal	0.29	0.9980	23.6	1.8	15.5	88±22	102±12	4.0	13.3
Tyr	0.005-5	External	1e6	0.9947	4.4	0.1	9.9	131±13	112±19	0.32	1.1
DOPAC	0.005-2.5	Internal	51.43	0.9991	0.67	3.3	14.1	69±17	96±9	0.33	1.1
HVA	0.050-5	External	5e4	0.9953	49.8	6.0	8.7	133±20	115±18	3.3	10.9
3-MT	0.005-2.5	Internal	1.82	0.9997	0.53	3.3	10.8	102±9	103±1	0.27	0.89
NE	0.005-2.5	Internal	1.97	0.9999	12.0	1.1	6.7	105±17	118±13	1.64	5.46

Figure legends

Figure 1. Detailed steps of the extraction and cleanup procedures under study. ISM: internal standard mixture; FA: formic acid; IPA: 2-propanol.

Figure 2. MRM (multiple reaction monitoring) chromatograms of the ten monoaminergic neurochemicals from a solvent mixed-standard solution (2 ng mL^{-1}) using the Acquity UPLC BEH Amide column.

Figure 3. Levels of monoaminergic neurochemicals in three regions of the adult zebrafish brain. The levels of the catecholaminergic neurotransmitters dopamine (DA) and norepinephrine (NE), as well as the serotonergic neurochemicals L-tryptophan, serotonin (5-HT) and 5-HIAA have been determined in adult zebrafish anterior (yellow), middle (magenta) and posterior (green) brain regions. Data are presented as mean ± standard error (SE). Different letters indicate significant ($P< 0.05$) differences following one-way ANOVA and Tukey's multiple-comparison test.

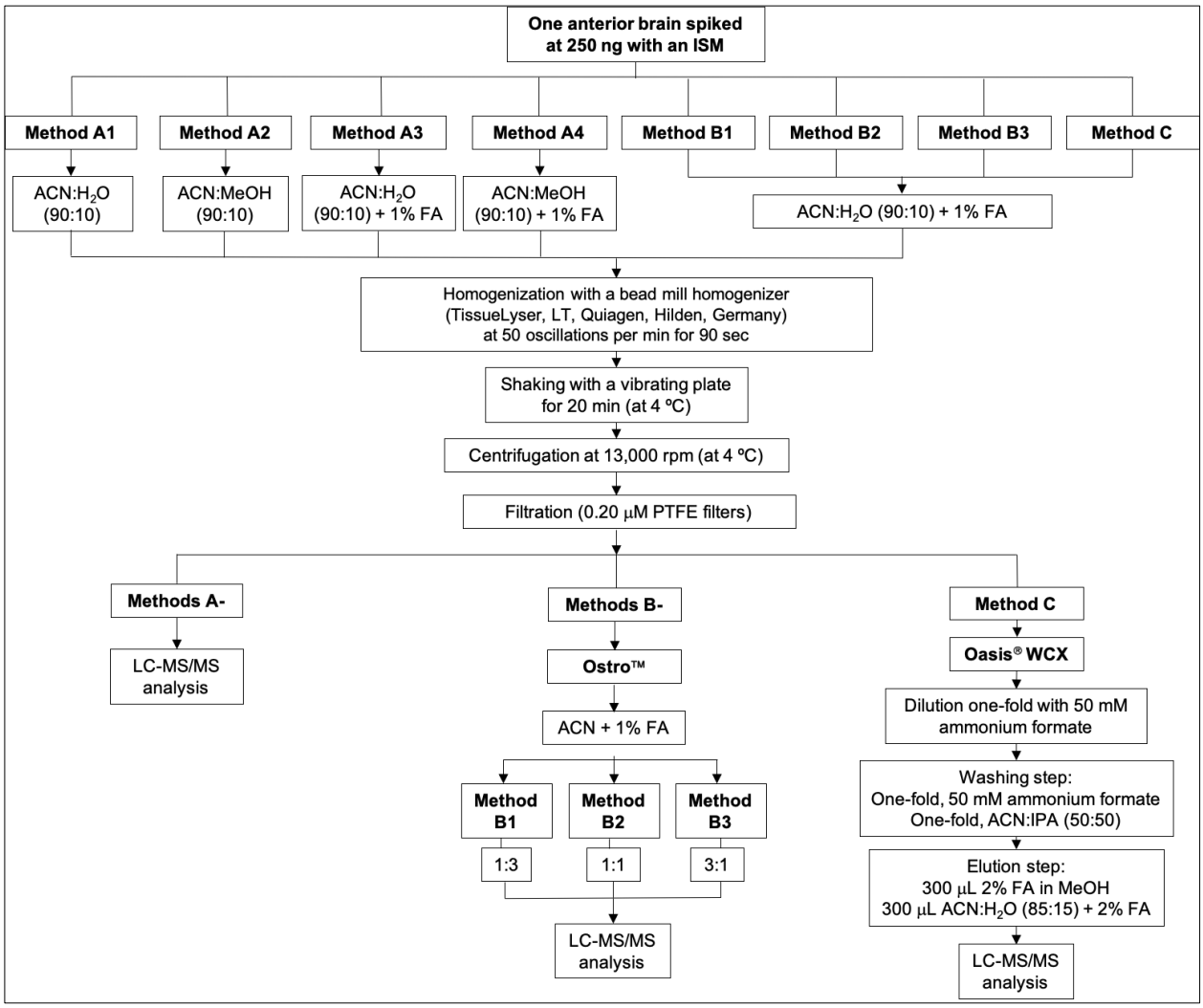


Figure 1. Detailed steps of the extraction and cleanup procedures under study. ISM: internal standard mixture; FA: formic acid; IPA: 2-propanol.

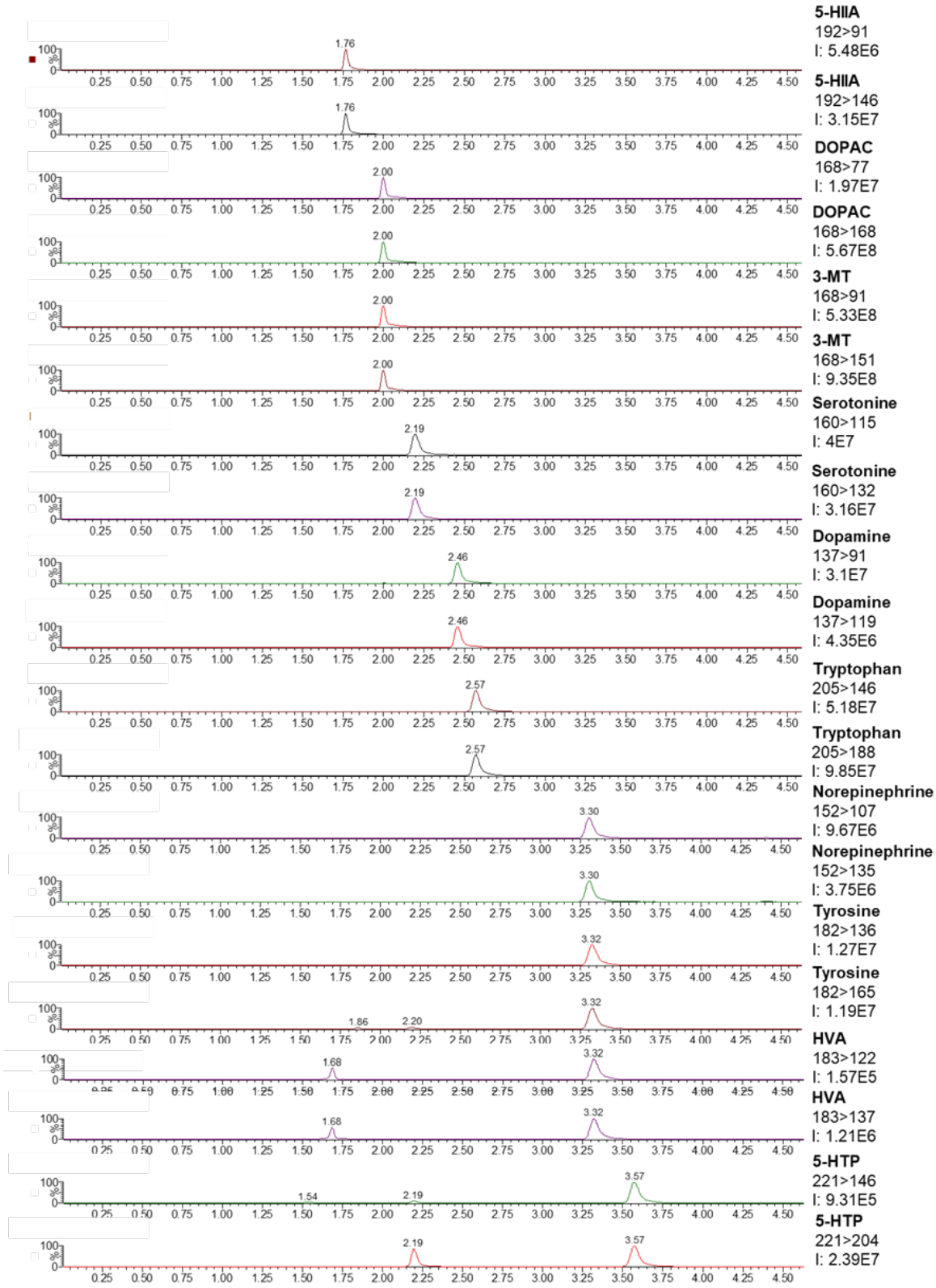
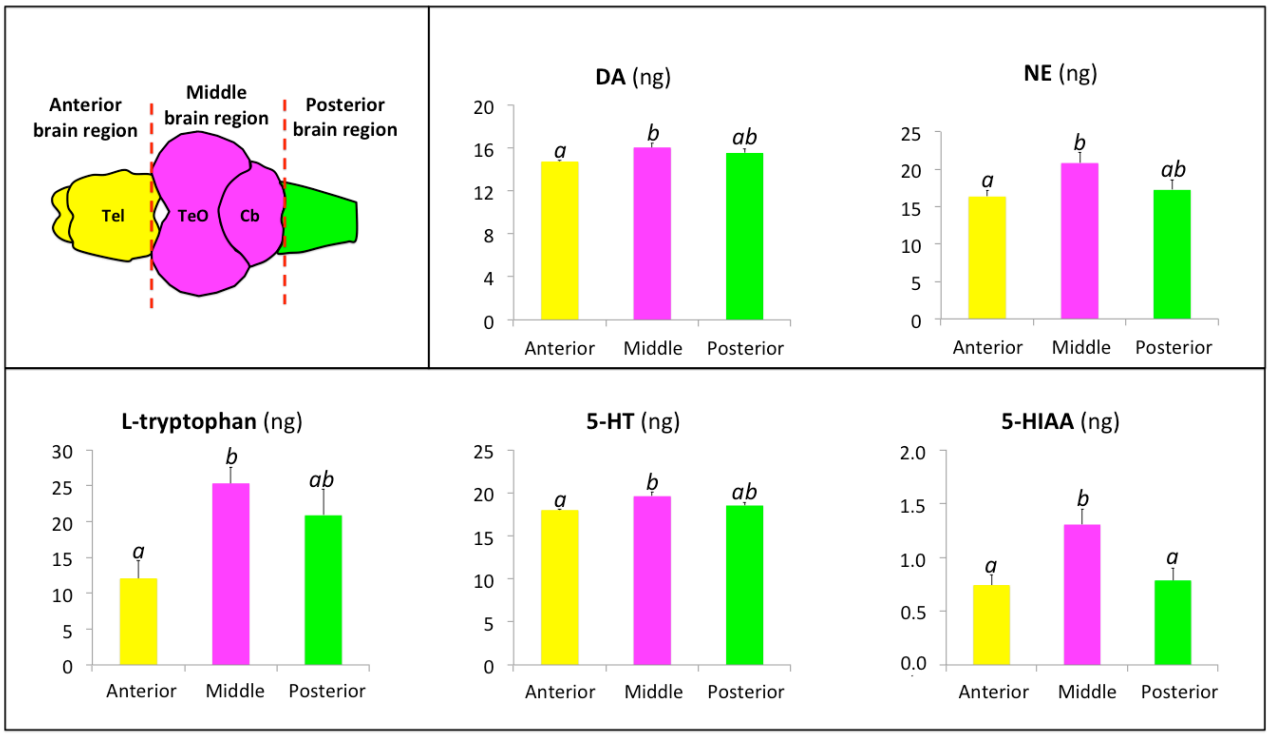


Figure 2. MRM (multiple reaction monitoring) chromatograms of the ten monoaminergic neurochemicals from a solvent mixed-standard solution (2.5 ng μL^{-1}) using the Acquity UPLC BEH Amide column (150 mm x 2.1 mm ID, particle size 1.7 μm).



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