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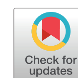

Heart-cutting two-dimensional liquid chromatography coupled to quadrupole-orbitrap high resolution mass spectrometry for determination of *N,N*-dimethyltryptamine in rat plasma and brain; Method development and application

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ABSTRACT

The orthogonal heart-cutting liquid chromatography (LC) modes coupled to high-resolution tandem mass spectrometry (HRMS/MS) provide a number of possibilities to enhance selectivity and sensitivity for the determination of targeted compounds in complex biological matricies. Here we report the development of a new fast 2D-LC-(HRMS/MS) method and its successful application for quantitative determination of the level of plasma and brain *N,N*-dimethyltriptamine (**DMT**) using α-methyltryptamine (**AMT**) as internal standard in an experimental model of cerebral ischemia/reperfusion using **DMT** administration. The 2D-LC separation was carried out by a combination of hydrophilic interaction liquid chromatography (HILIC) in the first dimension followed by second-dimensional reversed-phase (RP) chromatography within a total run time of 10 min. The enrichment of HILIC effluent of interest containing **DMT** was performed using a C18 trapping column. During method development several parameters of sample preparation procedures, chromatographic separation and mass spectrometric detection were optimised to achieve high **DMT** recovery (plasma: 90 %, brain: 88 %) and sensitivity (plasma: 0.108 ng/mL of LOD, brain: 0.212 ng/g of LOD) applying targeted analytical method with strict LC and HRMSMS confirmatory criteria.

Concerning rat plasma sample, the concentration of **DMT** before hypoxia (49.3–114.3 ng/mL plasma) was generally higher than that after hypoxia (10.6–96.1 ng/mL plasma). After treatment, the concentration of **DMT** in brain was elevated up to the range of 2–6.1 ng/g.

Overall, our analytical approach is suitable to detect and confirm the presence of **DMT** administered to experimental animals with therapeutic purpose in a reliable manner.

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1. Introduction

DMT is an endogenous hallucinogen and trace amine found in various tissues such as the brain, pineal gland and lung, and body fluids such as urine, cerebrospinal fluid and blood plasma in mammals [1–4]. **DMT** is produced by somatic cells under physiological conditions [1,3], and its concentration has been found to increase in response to pathophysiological homeostatic challenges (e.g. hypoxia or oxidative stress) [1,3]. Concurrently, **DMT** is best known as an indole alkaloid typically synthesised by plants like Psychotria viridis and Diplopterys cabrerana [5].

Although **DMT** was traced with gas-chromatography in body fluids of psychiatric patients and control subjects [3,4], its physiological role is still to be understood, in particular, because **DMT** undergoes rapid enzymatic degradation by monoamine oxidases (MAO) [3]. On the other hand, **DMT** has been implicated in neuroprotection at high concentration, either synthesised in the nervous tissue in response to hypoxia or oxidative stress, or originating from the lung and reaching the brain via the blood stream [1,6]. The putative neuroprotective action of **DMT** has been utilised in experimental models of cerebral or renal ischemia. **DMT** administered at supraphysiological concentration to experimental rodents resulted in a smaller cerebral infarct size [7] and promoted cell survival in a

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cell culture of human cerebrocortical neurons exposed to hypoxia [8].

Early researches of **DMT** focused primarily on the psychological and pharmacological effects of consuming ayahuasca [5]. During the last 60 years, many studies reported finding **DMT** and its metabolites in animal tissue and human urine, blood and cerebrospinal fluid using liquid chromatography (LC). Nowadays liquid chromatography–tandem mass spectrometry (LC–MS/MS) is one of the most widely used bioanalytical methods for quantification of small molecules in biological samples. It is due to its high sensitivity and high selectivity, thus being able to provide more reliable data than the LC technique itself, which is also true of the analysis of **DMT** [9], only a few studies reported the presence of **DMT** in mammalian tissue; for instance, in pineal gland microdialysate of rat [2], rat kidney, lung, liver, brain, human lung and rabbit liver [10]. According to certain viewpoints, detection of endogenous **DMT** is not relevant when it is exogenously administered due to the dissimilar concentration range [11].

The ultimate purpose of our upcoming study is to evaluate the neuroprotective action of **DMT** in an experimental model of cerebral ischemia/reperfusion, aggravated by transient anoxia and recurrent spreading depolarisations. Here, we set out to establish a novel, highly sensitive analytical tool to determine the concentration of endogenous **DMT** in our model and to measure the accumulation of intravenously administered exogenous **DMT** in blood plasma and brain tissue.

In modern pharmaceutical analysis, quantitation of analyte or analytes of interest in complex biological matrices requires high selectivity and sensitivity. Nowadays, two-dimensional liquid chromatography (2D-LC) is one of the most powerful analytical tools to improve the quality of obtained data, especially in combination with HRMS/MS. The comprehensive and heart-cutting analyses are known to be the main techniques of 2D-LC. The heart-cutting mode is a targeted 2D technique, because only a selected eluent fraction (single heart-cutting) or fractions (multiple heart-cutting) are collected, trapped for second-dimensional separation [12–14].

Nowadays, the typical method utilised for determination of administered **DMT** in plasma and brain sample is reversed phase chromatographic separation coupled to tandem mass spectrometry using electrospray or atmospheric pressure chemical ionization in positive mode [15–18]. However, the high resolution accurate HRMS/MS method, in combination with orthogonal 2D-LC separation in comparison with one dimensional LC-HRMS/MS methods, has the ability to provide more reliable data. Furthermore, it gives the opportunity to use lower amounts of samples with similar or better limit of detection for **DMT**. The required amount of biological sample is a critical issue, especially in pharmacokinetics assays or when the available sample is limited for instance at a repeated sampling of blood from rat and mouse. Another key factor of method development is run time, which should be kept as low as possible. For determination of **DMT** in complex biological samples such as brain and plasma, the application of the gradient elution is essential, therefore the total run time of analysis increases by the time of column wash and equilibration. In the literature, the total run time of gradient LC–MS/MS analysis of **DMT** can be found in the range of 11.5–25 min in brain and plasma samples [15–18]. Thus, the main goals of this study were the development of a fast heart-cutting 2D-LC-HRMS/MS method and optimisation of sample preparation procedures for the quantitative analysis of **DMT** in rat brain and plasma using a relatively low amount of biological samples. The method thus developed was applied for the determination of **DMT** level in rat plasma and brain of experimental model of cerebral ischemia/reperfusion using **DMT** administration.

2. Materials and methods

2.1. Chemicals and standards

N,N-Dimethyltryptamine (**DMT**) standard with purity >98.5 % was obtain from Lipomed AG (Arlesheim, Switzerland). α-Methyltryptamine (**AMT**) with purity >99 % was kindly provided by the Department of Forensic Medicine, Faculty of Medicine, University of Szeged, Hungary, and used as internal standard (**IS**). Acetonitrile, ammonium formate, formic acid, methanol, water, (all LC–MS grade), 35 % ammonia solution, ethyl acetate (HPLC grade) and reagent grade perchloric acid (70 %) were purchased from VWR (Radnor, PA, USA). Isoflurane (Medicus Partner Ltd., Biatorbágy, Hungary) for in vivo preparations, the anaesthetic isoflurane was produced by CP-Pharma (Handelsgesellschaft mbH, Germany); atropine and lidocaine were purchased from Egis Pharmaceuticals Ltd. (Budapest, Hungary); EDTA and pargilin from Sigma-Aldrich, (St Louis, MO, USA); chloral hydrate from Molar Chemicals Ltd. (Budapest, Hungary), and sterile physiological saline from B. Braun AG, (Melsungen, Germany).

2.2. Animals and tissue samples

The experimental procedures were approved by the National Food Chain Safety and Animal Health Directorate of Csongrád County, Hungary (license No. XXXII./2015). The procedures were performed according to the guidelines of the Scientific Committee of Animal Experimentation of the Hungarian Academy of Sciences (updated Law and Regulations on Animal Protection: 40/2013. (II. 14.) Gov. of Hungary), following the EU Directive 2010/63/EU on the protection.

Young 3 months old, male Sprague-Dawley rats (Charles River Laboratories, 328 ± 18 g) were used in this study. Standard rodent chow and tap water were supplied ad libitum. The animals were housed under constant temperature, humidity and lighting conditions (23 °C, 12:12 h light/dark cycle, lights on at 7 a.m.).

We set out to determine the blood plasma and brain concentration of **DMT** synthesised by tissues in response to ischemia/hypoxia (i.e. endogenous **DMT**), and **DMT** content of blood plasma and brain tissue after **DMT** administered to the animals by intravenous (i.v.) infusion (i.e. exogenous **DMT**), with the ultimate purpose to achieve neuroprotection against cerebral ischemia/hypoxia.

2.3. Surgical procedures and collection of samples

For endogenous **DMT** measurements, rats were injected intraperitoneally (i.p.) with pargilin (50 mg/bwkg, Sigma-Aldrich, USA), an effective monoamine-oxidase (MAO) inhibitor, a day prior to and on the day of the experiments. For the surgical procedures, animals were anaesthetised with 1.5–2 % isoflurane in N_2O:O_2 (3:2) and were allowed to breathe spontaneously through a head cone during the experiment. Body temperature was kept at 37.2 °C by a feedback-controlled heating pad (Harvard Apparatus, Holliston, MA, USA). Atropine (0.1 %, 0.05 mL; i.m.) was administered as premedication in order to avoid the production of airway mucus. The left femoral artery was cannulated for blood sampling, and the adjacent femoral vein was cannulated for drug administration. For the later initiation of incomplete global forebrain ischemia (n = 4), a midline incision was made in the neck, and each common carotid artery was carefully looped around with a surgical thread. Lidocaine (1 %) was used for local anaesthesia. Control rats were sham-operated (n = 3). All rats were fixed by their head in a prone position in a stereotaxic apparatus.

After a baseline period (5–10 min), cerebral ischemia was induced by the occlusion of both common carotid arteries. In half

an hour after ischemia onset, the insult was aggravated by hypoxia achieved by the complete withdrawal (5 min, endogenous **DMT** measurement) or controlled withdrawal (1 min, exogenous **DMT** measurement) of O_2 from the anaesthetic gas mixture. The complete withdrawal of O_2 terminated the experiments, while the controlled withdrawal of O_2 was followed by reoxygenation and the prompt release of the carotid arteries to allow cerebral reperfusion for another hour.

In rats prepared for the measurement of exogenous **DMT**, the administration of **DMT** (1 mg/bwkg/h in physiological saline) or its vehicle (physiological saline) started upon ischemia induction through the femoral vein, with the aid of a microinjection syringe pump (CMA/100 Micro Injection Pump, Carnegie Medicine, Stockholm, Sweden). The i.v. infusion of **DMT** was continuous and maintained until the end of the experiment.

For the determination of the endogenous **DMT** content of blood plasma and brain tissue, 5 mL arterial blood was collected immediately after the 5-min withdrawal of O_2. The full blood was centrifuged promptly in an Eppendorf tube (5000 g, 5 min, 4 °C, Heraeus Fresco 17 Microcentrifuge, Thermo Scientific, Waltham, MA, USA) coated with EDTA (ethylenediaminetetraacetic acid), and supernatant plasma (1 mL) was separated into another Eppendorf tube. The brains were removed and then, after snap-frozen in liquid nitrogen, were stored together with the plasma at −80 °C until further processing.

For the measurement of blood plasma and brain tissue concentration of exogenous **DMT**, blood samples were collected through the femoral artery at baseline (i.e. prior to ischemia onset), under ischemia, after hypoxia and under reperfusion. The animals were transcardially perfused with ice-cold physiological saline under deep chloral hydrate anaesthesia (5 %, i.p., 500 mg/bwkg) at the end of the experimental protocol to eliminate any blood from the brain. The brains were removed and snap frozen in liquid nitrogen, then stored at −80 °C until further processing. Blood samples were processed as described above and yielded 0.2 mL plasma.

2.4. Sample preparation of rat plasma samples

Prior to extraction of plasma, 5 μL **IS** (200 ng/mL in methanol) and 5 μL methanol was added to 50 μL of plasma sample. After vortex, 50 μL of 5.3 v/v% ammonia solution and 300 μL ethyl acetate were added followed by 30 s vortex and shaking for 10 min at room temperature (250 rpm, orbital, Dual-Action Shaker KL 2, Edmund Bühler, Bodelshausen, Germany). Then the mixture was rested for 5 min on ice. The sample was centrifuged at 4 °C for 12 min at 21,000 *g* (Universal 320 R, Hettich, Tuttlingen, Germany). 250 μL of the upper phase was collected and the lower phase was re-extracted with 300 μL of ethyl acetate. After centrifugation, 300 μL of the upper phase was combined with the first portion of aliquot and was dried under nitrogen at room temperature. For 2D-LC-HRMS/MS measurements, the dried extracts were dissolved in 100 μL methanol.

2.5. Sample preparation of rat brain samples

Rat brain tissue was homogenized based on the work by Kärkkäinen [10]. The weighed brain sample (1 g tissue, 7 mL ice-cold 0.1 M $HClO_4$) was added into 12 mL tubes. The sample was sonicated on ice with Biologics Model 150 V T ultrasonic homogenizer (BioLogics, Inc., Manassas, VA, USA) for 3 × 1 min using medium power setting on the micro-tip probe and an 80 % pulse. After centrifugation at 9000*g* for 12 min at 4 °C (Universal 320 R, Hettich, Tuttlingen, Germany), 5 μL IS (200 ng/mL in methanol), 5 μL methanol and finally 200 μL of 8 % ammonia solution were added to 400 μL brain homogenate. After vortex, 500 μL ethyl acetate was added, followed by 30 s vortex and shaking for 10 min at room temperature (250 RPM, orbital, Dual-Action Shaker KL 2, Edmund Bühler, Bodelshausen, Germany). Then the mixture was rested for 5 min on ice. The sample was centrifuged at 4 °C for 12 min at 21,000*g* (Universal 320 R, Hettich, Tuttlingen, Germany). 350 μL of the upper phase was collected and the lower phase was re-extracted with 500 μL of ethyl acetate. After centrifugation, 500 μL of the upper phase was combined with the first portion of the aliquot followed by drying under nitrogen at room temperature. For the 2D-LC-HRMS/MS measurements, the dried extracts were dissolved in 100 μL methanol.

2.6. Preparation of samples for calibration and validation

1.00 mg/mL stock solutions of **DMT** and **AMT** were prepared by dissolving approximately 1 mg aliquots of each component in methanol. The stocks were used for the preparation of working calibration standards. Eight **DMT** working calibration standard solutions were prepared in methanol. For external calibration, the sample preparation was similar to that of the treated samples, except that the sample was spiked with 5 μL of the given concentration of **DMT** instead of 5 μL methanol. Thus, the final volumes were the same in the calibration samples and the treated samples. External calibration curves using internal standard were used with the following calibration points of **DMT** (0, 0.2, 2, 10, 20, 40, 100 and 200 ng/mL plasma) in plasma and (0, 0.175, 1.75, 8.75, 17.5, 35, 87.5, 175 and 350 ng/g) in brain. Each calibration point was injected in replicate and repeatability was evaluated for the retention times of **DMT**.

The limit of detection (LOD) and limit of quantification (LOQ) were calculated, respectively, on the basis on the standard deviation (SD) and the slope (S) of the calibration curve.

$$LOD = \frac{3.3 \times SD}{S}$$

$$LOQ = \frac{10 \times SD}{S}$$

Accuracy and precision was determined by analysing samples at “Low” (10 ng/mL in plasma and 17.5 ng/g in brain), “Mid” (40 ng/mL in plasma and 35 ng/g in brain) and “High” (200 ng/mL in plasma and 350 ng/g in brain) concentrations in three analytical runs.

Carry over was assessed by injecting a blank sample after injecting a high concentration of analyte (plasma: 200 ng/mL, brain: 350 ng/g).

To evaluate the stability of **DMT** in plasma and brain samples in the autosampler (16 °C), calibration solutions (20 ng/mL in plasma and 35 ng/g in brain) were re-injected 24 h after the first injection and compared to the original concentrations. The results of the second runs were expressed as the percentage of their respective values in the first run.

2.7. 2D-LC-HRMS/MS conditions

The 2D-LC-HRMS/MS analysis was performed with Thermo Scientific Q Exactive Plus hybrid quadrupole-Orbitrap (Thermo Fisher Scientific, Waltham, MA, USA) mass spectrometer coupled to a Waters Acquity I-Class UPLC^{TM} (Waters, Manchester, UK) apparatus. The UHPLC system equipped with two binary solvent managers, an auto-sampler and a column manager with two six-port, two-position automatic switch valves. The eluent of the trap column was diluted using a Knauer HPLC-pump 64 (Knauer, Berlin, Germany) and a high-pressure stainless steel tee (IDEX, Oak Harbor, WA, USA). The first- and second-dimensional analytical columns were thermostatted in the column manager of the UHPLC system,

while the trap column was inserted into an L-7350 LaChrom column oven (Merck, Darmstadt, Germany).

In the final 2D-LC-HRMS/MS analysis, the first-dimensional chromatographic separation was carried out on a Kinetex HILIC column (150 × 2.1 mm, 2.6 μm, 100 Å, Phenomenex) protected by a HILIC guard column (4 × 3 mm, 2.6 μm, 100 Å, Phenomenex), while the second-dimensional separation was performed on a Luna Omega Polar C18 column (50 × 2.1 mm, 1.6 μm, 100 Å, Phenomenex). For the enrichment of the analytes, a Luna C18 trap column (20 × 2 mm, 5 μm, 100 Å, Phenomenex) was applied and connected through two six-port valves (Fig. 1).

During method development, the following chromatographic columns were tested in the first dimension: Luna HILIC (150 × 3 mm, 3 μm, 200 Å, Phenomenex), Luna Omega Sugar (100 × 2.1 mm, 3 μm, 100 Å, Phenomenex), Luna NH_2 (150 × 2 mm, 3 μm, 100 Å, Phenomenex) and Kinetex C18 (50 × 2.1 mm, 2.6 μm, 100 Å, Phenomenex). Columns tried in the second dimension were Luna Omega PS C18 (50 × 2.1 mm, 1.6 μm, 100 Å, Phenomenex), Acquity UPLC BEH C18 (50 × 2.1 mm, 1.7 μm, 130 Å, Waters) and CORTECS UPLC C18+ (50 × 2.1 mm, 1.6 μm, 90 Å, Waters). By the scheduled switching of the six-port valves, the dilute mobile phase from the HILIC column in first dimension was trapped on Luna C18 enrichment column (Fig. 1). For the prevention of the elution of targeted compounds from the enrichment columns during the trapping process, the diluter pump delivered aqueous ammonia solution (pH 10.2) at 3 mL/min flow rate into the static mixing tee, which was connected to a HILIC column and a six-port valve. The temperature of trap columns was maintained at 50 °C (Fig. 1).

In the first dimension, a 10 μL sample was injected with the Partial Loop With Needle Overfill injection mode on a Kinetex HILIC column using the programmed gradient of eluent *A* (50 mM ammonium formate solution) and eluent *B* (0.1 % formic acid in acetonitrile). The column temperature was set at 50 °C during the analysis. In the second dimension, eluent *A* and *B*, respectively, were 0.1 % formic acid solution and 0.1 % formic acid in acetonitrile. The mobile phase washed the substances trapped on a Luna C18 enrichment column onto a Luna Omega Polar C18 column. The second-dimensional column was maintained at 50 °C. The gradient programs of both dimensional separations with the two six-port valve positions are detailed in Table 1.

The mass spectrometer was operated in positive-ion mode using a heated electrospray ionisation (ESI) source with the following conditions: capillary temperature 250 °C, S-Lens RF level 50, spray voltage 3.5 kV, sheath gas flow 45, sweep gas flow 2 and auxiliary gas flow 10 in arbitrary unit. In parallel reaction monitoring (PRM) with resolution of 17,500 (FWHM), the automatic gain control (AGC) setting was defined as 1×10^6 charges and the maximum injection time was set to 50 ms The width of the isolation window of precursor ion was 2 Da. Collision energies (CE) were optimised for respective transitions of **DMT** and **AMT** (Fig. 2). The additional main HRMS/MS parameters are summarized in Table 2.

During 2D-LC-HRMS/MS measurements, the eluate was passed into the ESI source only in the time range of 4.5–5.5 min in order to decrease the contamination of the MS. For the remaining time, the ESI source was rinsed with acetonitrile/water solution (90/10, v/v) at a flow rate of 0.1 mL/min by an auxiliary pump (Knauer HPLC-pump 64, Knauer, Berlin, Germany).

The LC system was controlled by MassLynx 4.1 SCN 901 (Waters, Milford, MA, USA). The control of the MS system and MS data acquisition was conducted by Xcalibur^{TM} 4.1 software (Thermo Fisher Scientific, Waltham, MA). GraphPad Prism 5 statistical software (GraphPad Software, San Diego, CA, USA) was used for drawing column diagrams, boxplots and point graphs, and the calculation of paired t-test.

3. Results and discussion

3.1. Development of heart-cutting 2D-LC-HRMS/MS

During analytical method development several parameters were tested in both dimensions and trapping procedure such as mobile phase composition, column temperature, gradient steepness, flow rate, type and length of column. For ESI-HRMS/MS detection of **DMT** and **AMT**, the appropriate transitions (quantifiers, qualifiers ions) were selected and the related collision energies and ESI parameters were optimised (Fig. 2).

The main aim of optimisation of HILIC separation of **DMT** and **AMT** (internal standard) was to achieve proper retain and peak shape of **DMT** and **AMT**, which is feasible involving equilibration time within maximum 10 min as a planned complete runtime of online 2D measurements. For the optimisation of HILIC, narrow-bore columns (3 and 2.1 mm) with length of 100 mm and 150 mm, packed with totally porous or core-shell particles were compared with different solid phases. The presence of water at a minimum quantity of 3 % in the initial mobile phase composition is essential in HILIC. By varying its concentration, the retention mechanism and separation can be influenced in both isocratic and gradient elutions [19].

The MS-compatible ammonium formate as mobile phase modifier was selected at 50 mM concentration applying acetonitrile as the organic content of the mobile phase. The flow rate is also a critical parameter in the first dimension, since it must be kept as low as possible, thus assisting the trapping of the investigated analytes. Unfortunately, the undesired effect of low flow rate is peak broadening. In our case, a flow rate of 0.3 mL/min provided an acceptable compromise. For appropriate column selection, four HILIC columns were tested (Luna HILIC, Kinetex HILIC, Luna Omega Sugar, Luna NH_2) using a 50 mM ammonium formate aqueous solution/0.1 % formic acid in acetonitrile (90/10, v/v) mobile phase composition for 6 min. A comparison of the separation performance of **AMT** and **DMT** on the four HILIC columns revealed that application of Luna Omega was disadvantageous for both retention and peak shapes of **DMT** (1.35 min) and **AMT** (1.40 min). Similarly, when Luna NH_2 was used, **AMT** gave an extreme peak broadening, whereas **DMT** (1.23 min) had a relatively low retention. In the case of Luna HILIC, significantly higher retention times were found for both **DMT** (3.89 min) and **AMT** (4.84 min) with a peak width of approximately 1 min. Interestingly, the elution order changed using Kinetex HILIC column, and the retention times of both analytes were higher than 6 min. Therefore, the eluent strength was increased to 50 mM ammonium formate aqueous solution/0.1 % formic acid in acetonitrile (85/15, v/v) resulting in 3.09 min and 2.72 min retention time for **DMT** and **AMT** and a peak width of approximately 0.5 min. Consequently, this composition was selected in the final method. Interestingly, the elution order changed using the Kintex HILIC column in comparison with those of the other three columns.

In the case of optimisation of second dimension, the primary aim was to obtain sharp, symmetric chromatographic peaks (high sensitivity) with close retentions for both compounds using gradient elution and keeping 10 min total run time of 2D analysis. Initially, five C18 columns were compared, using gradient elution (0–1 min 10 % B, 1–4 min 40 % B, where eluents A and B, were 0.1 % formic acid aqueous solution and 0.1 % formic acid in MeOH), respectively. Using CORTECS C18, Omega PS C18, and Kinetex C18, relatively high peak widths (~ 0.5 and 0.3 min for **DMT** and **AMT**) were observed. The application of BEH C18 and Omega C18 provided higher retention times and lower peak widths compared with those over other C18 columns. Finally, an Omega C18 microbore column with a particle size of 1.6 μm was selected for further optimisation procedures. The nature of organic solvent influenced both peak width and retention. For **DMT** and **AMT**, it could be concluded

Fig. 1. Flow scheme of the established 2D-LC/MS system in both valve positions: Position 1 (A) and Position 2 (B).

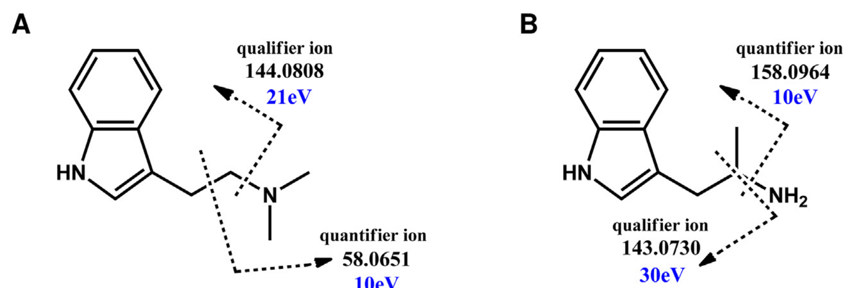

Fig. 2. The structure of **DMT** (A) and **AMT** (B) with assumed fragmentation cleavages, the exact mass of quantifier and qualifier ions and their optimised collision energy.

that ACN instead of MeOH gave lower retention, better peak shape and lower peak width. For obtaining proper retention within 10-min runtime including washing and re-equilibration of the column connected to trapping column in the second dimension, the temperature of the column, the flow rate and the gradient steepness were adjusted and synchronized with the HILIC method.

Table 1
The gradient programs of first and second dimension with the two six-port valve positions and analysis mode.

Gradient program of 1st dimension			Gradient program of 2nd dimension			Timetable of valves			Mode
Time (min)	B%	Flow rate (mL/min)	Time (min)	B%	Flow rate (mL/min)	Time (min)	Valve 1	Valve 2	
0.00	**85**	**0.3**	0.00	2	0.5	0.00	Position 2	Position 2	
						1.80	**Position 1**	**Position 1**	
			3.00	2	0.5				trapping
			3.10	2	0.3				
			3.50	**2**	**0.3**	**3.50**	**Position 2**	**Position 2**	
4.00	**85**	**0.3**							
4.50	50	1.0							analysis
			5.50	**100**	**0.3**				
			6.00	100	0.3				
			6.10	100	0.5				
6.50	50	1.0							
7.00	85	1.2							
						8.00	Position 1	Position 1	
						8.01	Position 2	Position 2	
			9.00	100	0.5				
			9.50	2	0.5	9.50	Position 1	Position 1	
						9.51	Position 2	Position 2	
9.90	85	1.2							
10.00	85	0.3	10.00	2	0.5				

Table 2
Main HRMS/MS parameters for **DMT** and **AMT** (internal standard) and retention times.

Analyte	Chemical formula	$[M+H]^+$	Product ion 1 (quantifier ion) m/z	Collision energy (eV)	Product ion 2 (qualifier ion) m/z	Collision energy (eV)	Retention time (min)
DMT	$C_{12}H_{16}N_2$	189.1386	58.0651	10	144.0808	21	4.99
AMT	$C_{11}H_{14}N_2$	175.1230	158.0964	10	143.0730	30	4.99

The online trapping of **DMT** and **AMT** from the eluate of the first-dimensional HILIC column in a 2D-LC system was performed by using C18 trap column. This highly orthogonal separation (HILIC × RP-LC) required to decrease the solvent strength of the effluent of the first-dimensional column in order to retain both analytes on the enrichment column. Fig. 1. demonstrates the experimental configuration of our 2D-LC-HRMS/MS system. The 2D interface between HILIC and RP columns consists of C18 trap column and two automatically operated two-position six-port valves. The dilution of the first-dimensional HILIC eluate was performed by applying a diluter pump connected to the column through a static mixing tee (Fig. 1). The selection of an appropriate diluter solvent and flow rate was crucial to enrich **DMT** and **AMT** on the trapping column with high efficiency. It can be explained by the high eluting power of eluate of HILIC separation in RP mode and, consequently, it needed to be diluted with a weak RP solvent. Initially, 0.1 % formic acid aqueous solution was used with a flow rate of 1 mL/min. However, it resulted in poor retention for both analytes on the trapping column (Fig. 1). Therefore, the effect of the flow rate of the diluting eluent on retention was investigated in the range of 1–3 mL/min. Fig. 3 shows that, by increasing the flow rate up to 3 mL/min, trapping efficiencies were only slightly improved. These negligible changes can be explained by the pK_a values of **DMT** (8.68) [20] and **AMT** (9.96) [21]. A comparison revealed that at low retention in the RP mode using of 0.1 % formic acid aqueous diluter solution with a pH of 2.7, the protonated forms of **DMT** and **AMT** were the dominant species. It seemed obvious that the retention of **AMT** and **DMT** on the trapping column can be enhanced by suppressing the polar cationic forms by increasing the pH of the diluter solvent. The increase of pH up to 10.2 in aqueous ammonia solution was an acceptable compromise considering the sufficient trapping of the two analytes and the pH stability of both the trapping and the Omega C18 columns. Fig. 3 demonstrates the dramatic improvement of trapping efficiency of **DMT** and **AMT** by the increase of pH of the diluter solvent. For **DMT**, higher enhancement was found, which could be explained by its lower pK_a value by ~1.3 units compared with that of **AMT**.

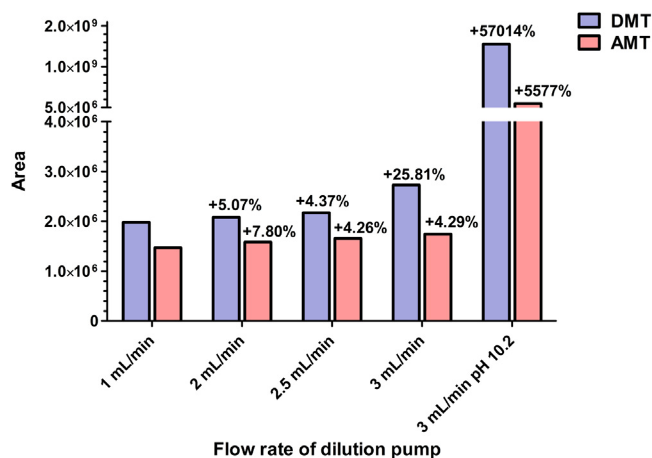

Fig. 3. The effect of the flow rate of diluting eluent to trapping efficiency of **DMT** and **AMT** during trapping of the heart-cut 1D eluate in the range of 1–3 mL/min (0.1 % FA), and 3 mL/min (ammonia solution at pH 10.2). The percentage increases compared to consecutive experiments are indicated by curved arrows.

Fig. 1 **(A)** displays the schematic diagram of our 2D-LC-HRMS/MS system in P̈osition 1″ status, where the C̈18 Trapčolumn was used to trap **DMT** and **AMT** from the dilute effluent of the HILIC column. During the trapping period, the gradient program of HILIC method was in isocratic section (Table 1). The system was kept in trapping configuration from 1.8 min to 3.49 min. At 3.50 min the switching valves were turned into P̈osition 2″ and thus C̈18 trapčolumn was connected to the RP analytical column as shown in Fig. 1 **(B)**. The particular design of the eluent gradient program was applied to wash out trapped analytes from C̈18 trapčolumn onto C18 analytical column, where the chromatographic separation occurred followed by HRMS/MS detection of **DMT** and **AMT**. Table 1 summarizes the final setup of events and gradient time programs in both dimensions. To keep the short 10 min 2D run time, the HILIC column was washed and equilibrated from 3.5 to 10 min, while the

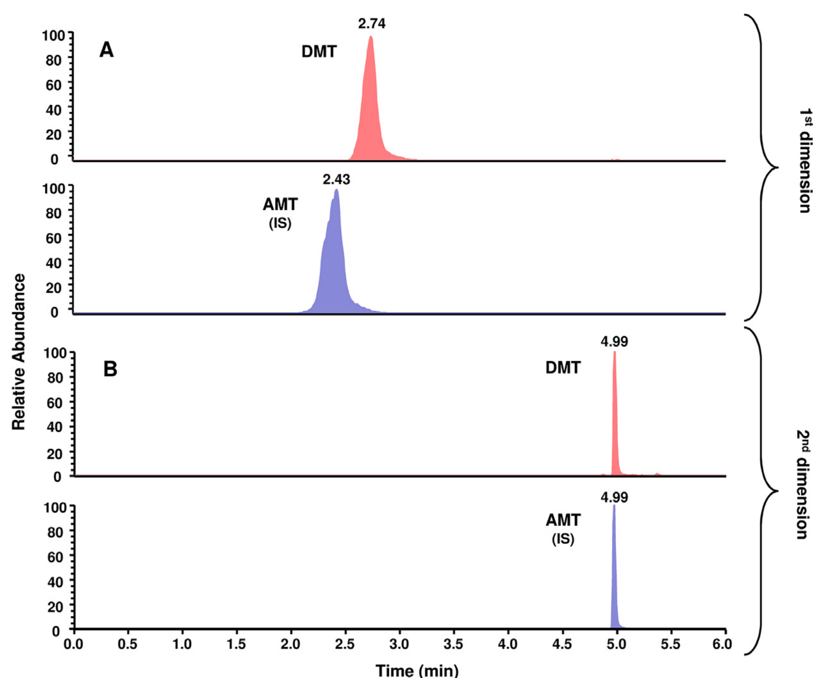

Fig. 4. Extracted ion chromatograms of quantifier ion transition of **DMT** and **AMT** detected by positive mode with 1D-HILIC method (A) and 2D-LC-HRMS/MS method (B) in standard solution.

trap and C18 columns were in the ranges of 8–10 min and up to 3.5 min in the next run using eluent and the flow rate gradient program in both dimensions. The obtained 10 min total run time was faster than various published one dimensional LC–MS/MS methods for **DMT** analysis in brain and plasma [15–18]. The elution profiles of **DMT** and **AMT** in the final 1D and 2D chromatograms of standard solutions are shown in Fig. 4 **(A, B)**.

3.2. Determination of extraction recovery, matrix effect, process efficiency and main validation parameters

Hence, the challenge of this work was to find a relatively simple sample preparation procedure, which is suitable for enrichment of **DMT** from rat plasma and brain samples. In the literature, several sample preparation methods related to **DMT** are reported [10,15–18,22,23]. Unfortunately, in many cases the main parameters, such as extraction recovery (RE), matrix effect (ME), process efficiency (PE), characteristics of the efficiency of a given method, were not presented. Labour-intensive and expensive solid-phase extraction procedures are widely employed nowadays for the enrichment of **DMT** from mammalian tissues [10,15,16]. The application of 2D-LC has an ability to decrease the negative matrix effect under analysis thereby providing an opportunity to simplify the sample preparation procedure. Therefore, the liquid–liquid extraction procedure seemed to be an obvious choice for the development of a new method. Without detailing the process optimisation of the sample preparation procedure, the use of ethyl acetate as organic solvent and a basic pH in sample solution helped to enrich **DMT** properly in the case of plasma and brain samples (Fig. 5 **(A, B)**). The relevant RE, ME and PE of the new method were determined in accordance with the procedure of Matuszewski et al. and Cappiello et al. [24,25]

Fig. 5 **(A, B)** illustrates the main indicators (RE, ME and PE) of the new analytical method for **DMT** and **AMT** in rat plasma (20 ng/mL) and brain (17.5 ng/mL). For **DMT**, the obtained 90 % of RE of our new sample preparation procedure in plasma was better with approximately 20–30 % than data in early studies [17,18]. The obtained high mean values of RE with really good repeatability (~ 10 % SEM) for **DMT** and **AMT** standard reveal that our new extraction methods are well-suited for both plasma and brain samples. The presence of strong ionisation enhancement during HRMS/MS detection resulted in a high positive ME value of **DMT** from plasma (161 %) and brain (174 %). The obtained high ME in plasma was much favourable than reported by Meyers (46 %) in 2014 [17]. However, by investigating ME of **AMT**, moderate ion suppression occurred resulting in a value of 87 % in brain (Fig. 5 **(B)**). Overall, the obtained favourable ME values for both compounds from a complex biological matrix clearly indicated the benefit of application of this 2D-LC-HRMS/MS method. In the case of **DMT**, the 145 % (plasma) and 153 % (brain) of PE, as resultants of values of RE and PE, lend evidence to the adequacy of the new method (Fig. 5 **(A, B)**).

Table 3
Main **v**alidation parameters of 2D-LC-HRMS/MS method.

	Plasma	Brain
Repeatability of retention time range (min)	4.99 ± 0.01	4.99 ± 0.01
LOD	0.108 ng/mL	0.212 ng/g
LOQ	0.328 ng/mL	0.642 ng/g
Accuracy Low (%)	111.7	111.6
Accuracy Mid (%)	89.6	88.5
Accuracy High (%)	102.6	100.9
Precision Low (%CV)	2.8	2.7
Precision Mid (%CV)	1.4	5.1
Precision High (%CV)	1.4	1.3
Carry over (%)	0.056	0.078
Stability (%)	−0.452	−0.500

Limit of detection (LOD), limit of quantification (LOQ) and percent coefficient of variation (%CV).

For brain and plasma samples, the main validation parameters of **DMT** are summarized in Table 3. After full optimization of the 2D method, good repeatability of the retention time of **DMT** with in the range of 4.98–5.00 min was achieved. During method development, the main issue was to keep the required volume of plasma as low as possible in order to decrease the negative effect of blood collection during surgical procedures [26]. For **DMT**, our 2D-LC-HRMS/MS method provided 0.108 ng/mL of LOD using 50 μL of plasma, which is significantly better than those reported in earlier studies, such as

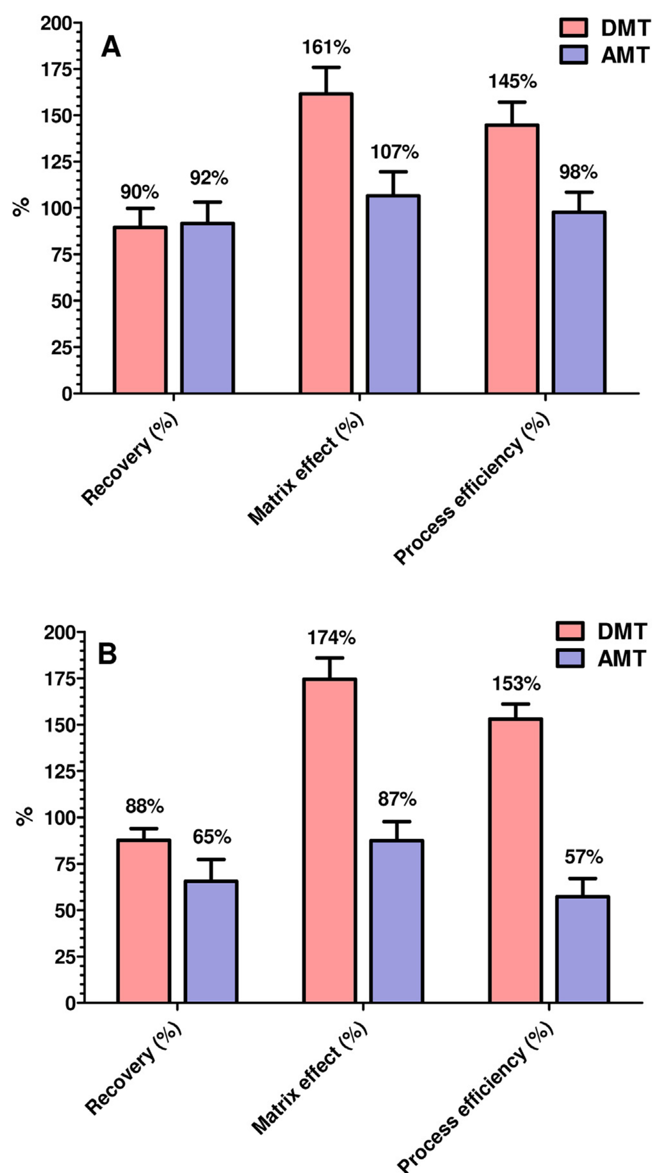

Fig. 5. Extraction recovery, matrix effect and process efficiencies for **DMT** (plasma: 20 ng/mL, brain: 17.5 ng/mL) and **AMT** (plasma: 20 ng/mL, brain: 17.5 ng/mL) standards from rat plasma (A) and rat brain homogenate (B). The extraction recovery and matrix effect percentage were calculated as follows: Extraction recovery (%) = $C/B \times 100$, Matrix effect (%) = $B/A \times 100$ and Process efficiency (%) = $C/A \times 100$, where A represents the average peak area of the standard solution, B represents the average peak area of the plasma or brain extract spiked at the same concentration of the standard and C represents the average peak area of a plasma or brain pre-spiked at the same concentration of the standard.

100 ng/mL using 500 μL plasma [17] and 0.45 ng/mL from 200 μL plasma [18]. Interestingly, Oliveira et al. reported 0.1 ng/mL of LOD for **DMT** in plasma [16]. However, the solid phase extraction procedure of their analytical method required 1000 μL of plasma. In brain sample the achieved LOD of **DMT** in our case was also better than that reported by Barker et al. (0.5 ng/g) [15].

The calibration curves (Figs. S13–S24) for **DMT** were linear (R2 > 99 %) in the range of 0.2–200 ng/mL plasma and 1.75–350 ng/g brain. Accuracy of our new method in three different concentration levels were determined within an acceptable interval of 12 % in plasma and brain samples. The precisions were found to be ≤2.8 % coefficient of variation (CV) in case of plasma and ≤5.1 % CV (brain). The obtained carry over of **DMT** was negligible (≤0.08 %) in plasma and brain.

3.3. Quantification of DMT in rat plasma and brain

In the first dimension of our orthogonal 2D method, HILIC mode was applied for the separation of **DMT** and **AMT** from plasma and brain matrix-derived endogenous components. In the method development process, an important consideration was to cut the peaks of interest from 1D to 2D in a narrow time window (1.8 min for 3.5 min), thus to keep the amount of undesirable exo- and endogenous matrix compounds in a low level in the second dimension. It is worth to be noted that the trapping process at basic pH also decreased the amount of compounds with acidic character with a chromatographic behaviour similar to those of **DMT** and **AMT** in HILIC condition. For these reasons, a relatively fast gradient method was applied in the second dimension. The obtained ME values confirmed the validity of the steep RP gradient program applied. Another advantage was that **AMT** as internal standard allotted with **DMT** as targeted compound at same retention time, but not in dead time, which is the chromatographic expectation of the standard calibration method towards an internal standard (Figs. S1–S4).

The developed method was applied to the quantification of **DMT** in rat plasma and brain tissue. The main aims of the application of this new 2D-LC-HRMS/MS method were to follow the plasma **DMT** level before ischemia onset, under ischemia, after hypoxia and under reperfusion, and determination of **DMT** concentration in related brain tissue after reperfusion. Therefore, the plasma and brain samples of preliminary experiments were used for optimisation of the sample preparation procedure and determination of the concentration range of external calibration using internal standards. A wide calibration concentration range was attempted for quantification of endogenous and exogenous **DMT** levels in a single run. To our knowledge, there exists only a single report from 2005 in which endogenous **DMT** was quantified in both rat brain and plasma using isoctratic LC–MS/MS method [10]. Unfortunately, we could not confirm the presence of endogenous **DMT** in rat brain and plasma. It is important to note, however, that our 2D-LC-HRMS/MS method provided more reliable data due to 2D-LC separation and accurate high resolution mass spectrometry detection with related strict LC and HRMSMS confirmatory criteria: specific retention time (4.99 min) with two characteristic precursor–product ion transitions using 50 ppm mass accuracy (quantifier ion at 58.0651 m/z and qualifier ion at 144.0808 m/z) and the ratio of the peak area of the quantifier ion and qualifier ion (2.1 ± 0.21) (Figs. S5–S12). Despite the fact that the quantifier ion was detected, but one the above-mentioned other parameters was not completed, the concentration of **DMT** was found to be lower than the limit of detection. In the final method, the calibration curve was optimised to measure exogenous **DMT** level, and **DMT** showed a good linearity range of 0.2–200 ng/mL plasma and 1.75–350 ng/g brain with R2 values higher than 0.99 (Figs. S13, S14).

The level of **DMT** in plasma samples of treated rats is illustrated in Fig. 6 **(A)**. All plasma samples collected before administration of **DMT** showed less than the detectable amounts of **DMT**. The highest concentration of **DMT** in the range of 49.3–114.3 ng/mL was observed in plasma for each animal before hypoxia. For animals 1, 3 and 4, the concentrations of **DMT** decreased significantly after hypoxia in plasma. After hypoxia to the end of the treatment, the concentration of **DMT** decreased to a mean value of 3.8 ng/mL, in line with the termination of **DMT** application.

Another question that remains to be answered is whether **DMT** accumulates to a detectable concentration in the brain. Thus, endogenous and exogenous **DMT** were analysed in brain tissue in both the control and treated animals. Similar to plasma samples, the endogenous **DMT** was not detectable in the brain of control animals. However, after treatment, the concentration of **DMT** changed in the range of 2–6.1 ng/g brain (Fig. 6 **(B)**).

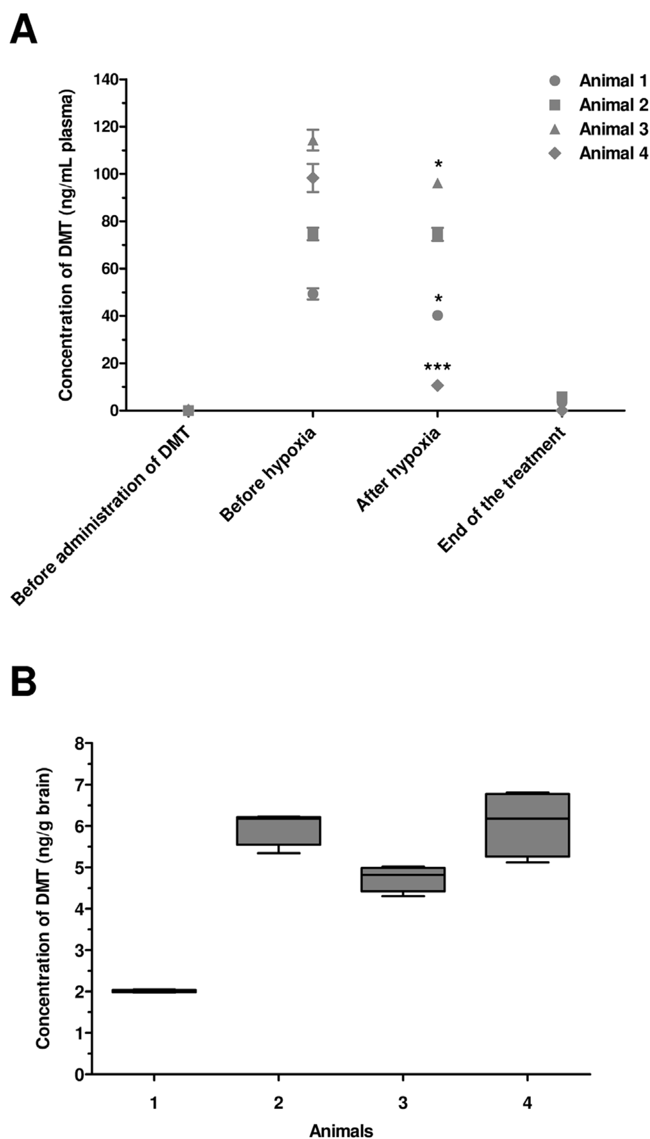

Fig. 6. The obtained concentration of **DMT** in plasma (A) and brain (B). Three repeated measurements were used for statistical analysis (*$p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$).

The concentrations of exogenous **DMT** measured are considered to be underestimated of the actual concentrations, because **DMT** must have been rapidly degraded during collecting biological samples [27], in particular, when harvesting brain tissue.

4. Conclusion

This paper describes the development and application of a fast heart-cutting 2D-LC-HRMS/MS method and related liquid-liquid extraction procedure for the analysis of **DMT**. By connecting orthogonal HILIC and RP chromatography through the use of RP trap column and high resolution MS/MS detection, a sensitive and selective analytical method was successfully utilised within total run time of 10 min for determination of the concentration of **DMT** in rat plasma and brain of experimental model of cerebral ischemia/reperfusion using **DMT** administration The developed and optimised plasma and brain matrix-related sample preparation protocols provided high extraction recoveries (~90 %) and high positive matrix effect (plasma: +61 %, brain: ~ +74 %) of **DMT**. During analysis, **DMT** was identified and confirmed by its specific retention time in the second dimension, two characteristic precursor–product ion transitions and the ratio of quantifier to qualifier mass transitions. External calibration using internal standard was applied to obtain accurate concentrations of **DMT** in plasma and brain samples. This heart-cutting 2D-LC-HRMS/MS method with new sample preparation procedure gave an opportunity to improve the LOD with the decrease of the required amount of biological samples.

Concerning exogenous **DMT**, to the best of our knowledge, this is the first attempt to confirm – with an analytical tool – the presence of **DMT** administered with therapeutical purpose in mammalian tissues. In summary, our analytical approach is suitable to detect and confirm the presence of **DMT** administered to experimental animals with therapeutic purpose. Further refinement of biological sample harvesting will provide more accurate determination of actual **DMT** concentrations.

Declaration of Competing Interest

Authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jpba.2020.113615.

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