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LC/MS/MS analysis of the endogenous dimethyltryptamine hallucinogens, their precursors, and major metabolites in rat pineal gland microdialysate

Steven A. Barker[a]*, Jimo Borjigin[b], Izabela Lomnicka[a] and Rick Strassman[c]

ABSTRACT: We report a qualitative liquid chromatography–tandem mass spectrometry (LC/MS/MS) method for the simultaneous analysis of the three known *N,N*-dimethyltryptamine endogenous hallucinogens, their precursors and metabolites, as well as melatonin and its metabolic precursors. The method was characterized using artificial cerebrospinal fluid (aCSF) as the matrix and was subsequently applied to the analysis of rat brain pineal gland-aCSF microdialysate. The method describes the simultaneous analysis of 23 chemically diverse compounds plus a deuterated internal standard by direct injection, requiring no dilution or extraction of the samples. The results demonstrate that this is a simple, sensitive, specific and direct approach to the qualitative analysis of these compounds in this matrix. The protocol also employs stringent MS confirmatory criteria for the detection and confirmation of the compounds examined, including exact mass measurements. The excellent limits of detection and broad scope make it a valuable research tool for examining the endogenous hallucinogen pathways in the central nervous system. We report here, for the first time, the presence of *N,N*-dimethyltryptamine in pineal gland microdialysate obtained from the rat. Copyright © 2013 John Wiley & Sons, Ltd.

Keywords: *N,N*-dimethyltryptamines; pineal gland; microdialysis; rat brain; LC/MS/MS

Introduction

In a recent review of 69 published studies reporting the detection of purported endogenous hallucinogens [*N,N*-dimethyltryptamine (DMT); 5-hydroxy-DMT (HDMT, bufotenine); 5-methoxy-DMT (MDMT)] in humans (Barker *et al*., 2012), it was concluded that compelling mass spectral evidence exists for the confirmation of their presence in certain human biological fluids [cerebrospinal fluid (CSF; DMT and MDMT), blood (DMT and HDMT) and urine (DMT and HDMT)]. There is as yet no definitive information as to the possible normal or pathophysiological roles of DMT, HDMT or MDMT in humans or other species owing, in part, to the lack of comprehensive methods to detect and unequivocally confirm the presence of these compounds in biological tissues and fluids (Barker *et al*., 2012). Methodology to adequately assess their synthesis and turnover, simultaneously monitoring their precursors and metabolites, is also lacking.

Original interest in endogenous hallucinogens, and DMT in particular, was motivated by the hypothesis that these compounds had a biochemical role in the heterogeneous disease state of psychosis, especially schizophrenia (for a review see Barker *et al*., 1981a, Barker *et al*., 2012). More recently, interest in DMT has been renewed owing to its characterization as a ligand for the sigma-1 (Fontanilla *et al*., 2009; Su *et al*., 2009) and trace amine receptors (Su *et al*., 2009). Recent studies concerning the enzyme responsible for the biosynthesis of these compounds have also drawn further attention. Although the enzyme for the synthesis of the DMTs, indole-*N*-methyltransferase (INMT), was not thought to occur to any significant extent in brain (Thompson and Weinshilboum, 1998; Thompson *et al*., 1999), studies have now shown its presence in the central nervous system, including the pineal gland (Cozzi *et al*., 2011), motor neurons in the spinal cord (Mavlyutov *et al*., 2012; Cozzi *et al*., 2011) and in the retina (Cozzi *et al*., 2011). While the enzyme is present in these tissues, there has yet to be a definitive determination of whether DMT is actually synthesized in these tissues and, if so, how it is utilized or released from the tissues under normal or altered physiological conditions.

* Correspondence to: S. A. Barker, Department of Comparative Biomedical Sciences, School of Veterinary Medicine, Louisiana State University, Baton Rouge, LA 70803, USA. E-mail: sbarker@vetmed.lsu.edu

[a] Department of Comparative Biomedical Sciences, School of Veterinary Medicine, Louisiana State University, Baton Rouge, LA, 70803, USA

[b] Departments of Molecular & Integrative Physiology[1] and Neurology[2], University of Michigan Medical School, Ann Arbor, MI, 48109, USA

[c] Department of Psychiatry, University of New Mexico School of Medicine, Albuquerque, and Cottonwood Research Foundation, Gallup, New Mexico, USA

Abbreviations used: 2MTHBC, 2-methyl-1,2,3,4-THBC; CSF, cerebrospinal fluid; d_4-MDMT, $\alpha,\alpha,\beta,\beta$-tetradeutero-5-methoxy-*N,N*-dimethyltryptamine; DMT, *N,N*-dimethyltryptamine; DMTNO, DMT-*N*-oxide; HDMT, 5-hydroxy-DMT; HIAA, 5-hydroxy-IAA; HNATA, 5-hydroxy-*N*-acetyl-TA; HNMT, 5-hydroxy-*N*-methyl-TA; HTA, 5-hydroxy-TA; HTHBC, 6-hydroxy-THBC; HTRP, 5-hydroxy-tryptophan; IAA, indol-3-acetic acid; INMT, indole-*N*-methyltransferase; MAO, monoamine oxidase; MDMT, 5-methoxy-DMT; MIAA, 5-methoxy-IAA; MNMT, 5-methoxy-*N*-methyl-TA; MTA, 5-methoxy-TA; MTHBC, 6-methoxy-THBC; NMT, *N*-methyl-TA; TA, tryptamine; THBC, 1,2,3,4-tetrahydro-β-carboline; TRP, tryptophan.

Given the reported expression of INMT in the mammalian pineal gland, the binding of DMT to the sigma-1 and trace amine receptors, and the necessity for more comprehensive analytical methodology to begin to assess the possible function of the DMTs *in vivo*, we undertook to develop a protocol to screen for the presence of the DMTs, their precursors and metabolites in mammalian body fluids and tissues. We describe here the application of our method to rat pineal gland microdialysates using liquid chromatography–tandem mass spectrometry (LC/MS/MS) for the qualitative analysis of the three known endogenous hallucinogens and of 20 compounds that constitute most of their known precursors and major metabolites (Fig. 1), as well as melatonin and its biochemical precursors. The method was developed in artificial CSF (aCSF), which was used as the pineal gland dialysate. The method described uses positive ion electrospray ionization, monitoring the protonated molecular ion ($M+1^+$) of each targeted analyte and corresponding fragment ions (multiple reaction monitoring). It also applies stringent analytical criteria for the detection and confirmation of these compounds in this matrix, including exact mass determination using high-resolution MS. The developed protocol was applied to pineal gland-aCSF-microdialysates obtained from freely moving rats. These studies revealed, for the first time, the presence of DMT in pineal gland microdialysate obtained from the rat.

Materials and methods

Animals

Adult (12 weeks of age), male, Wistar rats, weighing 300–350 g each, were obtained from Harlan Laboratories (Indianapolis, IN, USA) and were housed in light–dark conditions of 12:12 h for at least one week before experiments. Food and water was provided *ad libitum*. All animal procedures were approved by the University Committee on Use and Care of Animals at the University of Michigan.

Pineal microdialysis

Rats were implanted with linear microdialysis probes with a molecular weight cut-off of 13 kDa and a membrane length of 12–15 mm, manufactured in-house, as described previously (see Borjigin and Liu, 2008, for surgical techniques and probe preparation). The linear probe traversed both the pineal gland as well as superficial layers of occipital cortex on either side of the gland. The pineal location was ascertained in each rat by the presence of melatonin in the dialysates (Borjigin and Liu, 2008). Following a recovery period of 2–3 days, animals were placed in microdialysis chambers. The chambers consisted of enclosed animal housing units equipped with their own lighting, which was controlled by an on–off timer, and fitted with a venting fan. Sample collection was accomplished with the aid of a liquid swivel. Pineal microdialysis was performed with aCSF solution flowing continuously through the pineal gland at 2 μL/min for 2 h. All sample collections were performed during daylight hours. Two tubes of dialysate were collected, each of which contained 120 μL collected over 1 h (2 μL/min). The pineal dialysates were collected in microcentrifuge tubes on ice, capped and stored at −80 °C for 2–4 weeks prior to being shipped on dry-ice for further analysis. Samples experienced a single freeze–thaw cycle prior to analysis.

Analytical standards

The following compounds were obtained from a commercial source (Sigma Aldrich, St Louis, MO, USA), and were of the highest available purity (>98%): DMT, MDMT, HDMT, tryptamine (TA), *N*-methyl-TA (NMT), 5-hydroxy-TA (serotonin; HTA), 5-hydroxy-*N*-

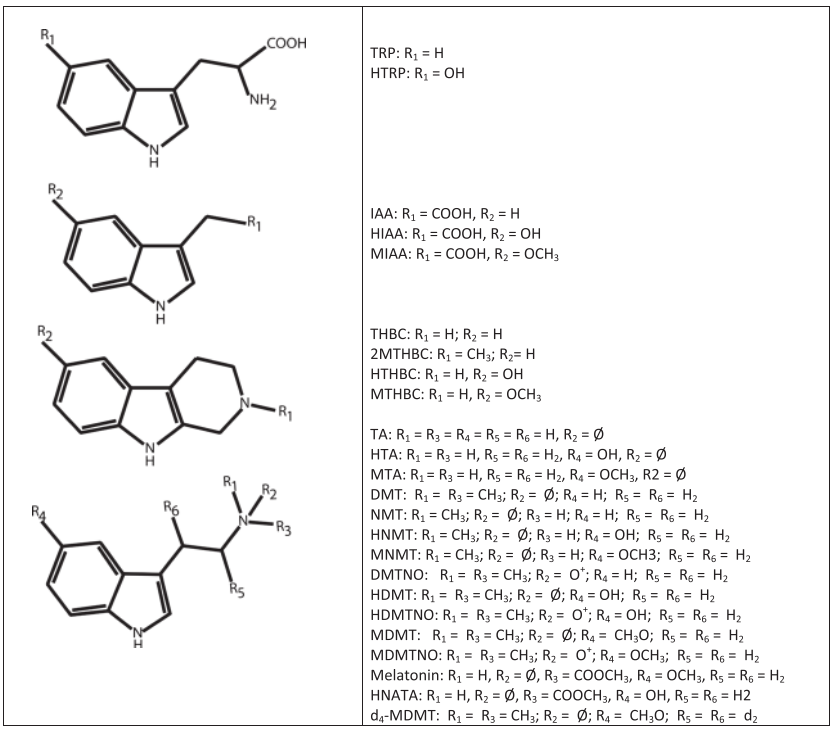

Figure 1. Compounds examined.

methyl-TA (HNMT), 5-methoxy-TA (MTA), 5-methoxy-*N*-methyl-TA (MNMT), melatonin, tryptophan (TRP), 5-hydroxy-tryptophan (HTRP), 5-hydroxy-*N*-acetyl-TA (HNATA), indol-3-acetic acid (IAA), 5-hydroxy-IAA (HIAA) and 5-methoxy-IAA (MIAA). The following compounds were synthesized (as noted) and their structures and purity confirmed (>98%) by LC/MS/MS: DMT-*N*-oxide (DMTNO), HDMTNO, MDMTNO (*N*-oxides were prepared as described by Fish *et al*., 1955), 1,2,3,4-tetrahydro-β-carboline (THBC), 2-methyl-1,2,3,4-THBC (2MTHBC), 6-hydroxy-THBC (HTHBC) and 6-methoxy-THBC (MTHBC) (β-carbolines were prepared by Pictet–Spengler reactions of the corresponding amines with formaldehyde according to the methods described by Ho and Walker, 1964). The internal standard was $\alpha,\alpha,\beta,\beta$-tetradeutero-5-methoxy-*N*,*N*-dimethyltryptamine (d_4-MDMT) and was kindly provided by Dr David Nichols (Purdue University). The structures of the target analytes are shown in Fig. 1.

Solvents and reagents

Solvents for liquid chromatography (LC) were obtained from Fisher Scientific (Fairlawn, NJ, USA) and were Optima grade (0.1% formic acid in water, 0.1% formic acid in acetonitrile).

Artificial CSF

Artificial CSF was prepared in Dr Borjigin's laboratory as defined by Alzet (http://www.alzet.com/products/guide_to_use/cfs_preparation.html) and contained NaCl (148 mM), KCl (3 mM), CaCl2·2H_2O (1.4 mM), MgCl2·6H_2O (0.8 mM), Na2HPO4·7H_2O (0.8 mM) and NaH2PO4·H_2O (0.2 mM). This solution was used in the analyses for preparation of standards, method blanks and spiked controls. The aCSF used for the mass spectral analyses was from the same batch as used to perform the microdialyses.

Instrumentation

Routine analyses were conducted using a Thermo (Thermo Electron North America, West Palm Beach, FL, USA) Quantum Access LC/MS/MS (triple quadrupole) system equipped with Accela 600 pumps and a multiplexed Accela open autosampler/injection system. Analyses were conducted using Thermo Scientific software (LC Quan 2.7.0 20, Xcalibur 2.2 SP1).

LC methods

Samples (aCSF dialysate obtained from rats, aCSF blanks, spikes and controls) were injected (30 μL) onto an Agilent (Santa Clara, CA, USA) Zorbax Eclipse Plus C_{18}, 3.0× 100 mm, 3.5 μm particle size column fitted with a 2.0 μm pre-filter (Grace Davison, Deerfield, IL, USA). Chromatography of the components was accomplished using a gradient LC program: solvent A = water–0.1% formic acid (Fisher Optima); solvent B = acetonitrile–0.1% formic acid (Fisher Optima); 0–2 min hold at 98% A–2% B, 2–6 min changing to 50% A–50% B with a 1 min hold, 7–8 min changing to 2% A–98% B with a 9 min hold, 17–18 min changing to 98% A–2% B and holding for 6 min before the next injection. The flow rate was 300 μL/min throughout the analysis. The waste divert valve was initiated from 0.0 to 1.3 min post injection and again at 15–24 min.

MS methods

The chromatography system was coupled to an ambient temperature electrospray ionization probe on the Quantum Access system. For analytes of interest, precursor-to-product ion transitions were established through direct infusion of neat standards of each compound into the ion source. Analytical standards (1 μg/mL) were dissolved in water or methanol and co-infused into the mass spectrometer with mobile phase to obtain optimal signal and fragmentation patterns/information (see Table 1). The sensitivity was optimized for each compound by manipulating collision energy to achieve the best signals.

Relevant MS settings for the analysis are shown in Table 1. The source conditions were set as follows: 4.0 kV ion spray voltage, 350 °C capillary temperature, sheath gas (N_2) pressure of 50 psi. The resolutions of Q1 and Q3 were set at unit mass. The dwell time was 10 ms for each multiple reaction monitoring transition. The Quantum Access system used a tune file, established prior to the analyses being initiated, for DMT as the tune compound.

Limits of detection and confirmation

Serial dilutions of standards in aCSF ($n = 4$) were analyzed using the Quantum Access LC/MS/MS system to determine the lowest limit of detection (LOD) and confirmation. All data were based on a 30 μL injection volume. Detection of the analyte was not considered positive unless all necessary confirmation criteria were met: retention time match vs in-run standard and relative retention time to internal standard (±1%), presence of predetermined fragment ions (two to four for each compound, not including the protonated molecular ion) and the agreement of ion ratios for the analyte vs in-run reference standards, within ±25% relative. Signals for each ion were not considered as detected unless they also exceeded 3 times baseline noise.

Specificity

Both blank aCSF and pineal dialysates from rats were examined for the presence of interferences. The target analytes were also examined to determine if any cross-talk would occur between the different compounds, since several of the analytes have the same molecular formula.

Sample preparation

No further preparation of the collected pineal dialysate, other than thawing, was required. The internal standard (d_4-MDMT), dissolved in 90:10 mobile phase, was added (10 μL) to each sample aliquot (50 μL) to give a final concentration of 10 ng/mL. Samples were placed into injection vials, capped, mixed and held at 4 °C in the dark in the injector tray during the entire period of the analysis (less than 8 h).

Additional confirmation methods

Analyses for confirmation of DMT employed different fragmentation conditions than previously reported, generating four fragment ions (58, 115, 143, 144 *m/z*) rather than the typical two ions (58 and 144 *m/z*) for DMT (Kärkkäinen *et al*., 2005; McIlhenny *et al*., 2011, 2012).

Additional confirmation data for DMT, as well as other compounds detected, were also obtained using a Thermo LC/

Table 1. MS parameters for analytes *and internal* standard, average retention times and limits of detection

Analyte	$[M+1]^+$	Product ion 1	CE1 (V)	Product ion 2	CE2 (V)	Product ion 3	CE3 (V)	Product ion 4	CE4 (V)	Limit of detection (ng/mL)	Retention time (min)
TRP	205.053	91.169	36	118.123	26	146.057	16			0.10	6.18
HTRP	221.053	106.204	32	134.095	24	162.048	16	203.996	7	0.30	5.50
IAA	176.043	77.214	39	103.140	32	130.098	15			0.40	8.03
HIAA	192.034	91.157	33	117.100	33	146.053	14			2.00	6.65
MIAA	206.036	117.101	33	145.043	28	160.036	15			0.06	7.87
THBC	173.072	77.206	38	143.040	29	144.067	13			0.05	6.66
2MTHBC	187.100	115.200	35	144.000	10					0.02	6.68
HTHBC	189.000	115.120	31	117.108	31	160.057	14			0.02	5.38
MTHBC	203.072	131.084	32	159.040	25	174.038	13			0.30	6.49
TA	161.092	115.110	33	117.106	24	144.075	10			0.30	6.31
HTA	177.078	115.124	27	117.120	28	160.049	11			0.40	5.45
MTA	191.079	130.089	35	159.035	21	174.046	10			0.40	6.47
NMT	175.000	132.000	10	144.000	10					0.02	6.45
HNMT	191.090	115.120	30	117.116	30	160.057	12			0.10	5.67
MNMT	205.089	130.113	38	159.038	24	174.053	12			0.03	6.47
DMT	189.000	58.000	13	144.080	16	115.175	40	143.134	30	0.03	6.56
HDMT	205.100	58.300	13	160.100	14					0.30	5.66
MDMT	219.100	58.100	26	174.100	13					0.25	6.70
DMTNO	205.100	117.100	27	144.100	16					0.02	6.80
HDMTNO	221.093	115.120	33	117.117	33	160.055	14			0.02	5.98
MDMTNO	235.127	130.107	43	159.051	27	174.054	13			0.08	6.82
Melatonin	233.089	130.110	41	159.049	26	174.052	13			0.05	7.68
HNATA	219.085	115.149	33	117.143	29	132.147	25	160.079	12	0.30	6.70
d_4-MDMT	223.200	60.360	15	134.160	43	178.110	15			—	6.60

CE, Collision energy.

Exactive OrbiTrap high-resolution mass spectrometer equipped with Accela 1250 pumps and a multiplexed Accela open autosampler/injection system. These analyses were performed on selected samples chosen on the basis of analyte response (intensity). Data were collected and processed using Thermo Xcalibur 2.2.0.48 and LC Quan 2.7.0 software. The OrbiTrap system was tuned using the exact mass of caffeine ($M+1^+$; 195.08037) as the reference/lock mass. Spray voltage was 3.8 kV, capillary temperature was 300 °C, sheath gas (N_2) was set to 25 psi, and the source heater temperature was 350 °C. The collision-induced-dissociation function was disabled. The same type of LC column was used for LC separation of the samples on the Thermo Exactive instrument experiments as was used for the experiments conducted on the Quantum Access, but a different LC program was utilized to further assist in confirmation: solvent A = water–0.1% formic acid (Fisher Optima); solvent B = acetonitrile–0.1% formic acid (Fisher Optima); 0–1 min hold at 90% A–10% B, 1–4 min changing to 50% A–50% B with a 1 min hold, 6–7 min changing to 2% A–98% B with a 9 min hold, 16–17 min changing to 90% A–10% B and holding for 6 min before the next injection. The flow rate was 300 μL/min throughout the analysis.

Analyte stability

All of the compounds examined were tested for stability in solution (artificial CSF) at −80 °C and 4 °C for up to 6 months and at ambient temperatures for at least 1 week in the dark. Absolute responses vs time were compared to determine changes in concentration.

Results and discussion

Compounds selected for analysis

The 23 compounds chosen for analysis represent a significant number of the known metabolites of tryptophan in biological species. They also represent the compounds in the specific pathways related to the potential formation and subsequent metabolism of the three known endogenous DMTs. The ability to monitor their precursors, metabolites, and related central nervous system indoleamines, as well as the three DMTs, simultaneously offers a significantly broader opportunity to assess the possible presence and role of these compounds individually and as a group than has ever been conducted before (Barker *et al*., 2012).

Each of the DMTs (DMT, HDMT, MDMT) is the biosynthetic product of INMT acting on their respective precursors, TA, HTA and MTA, yielding as intermediates NMT, HNMT and MNMT (for reviews see Barker *et al*., 1981a, 2012). The DMTs may also be enzymatically demethylated to yield the same mono-*N*-methylated compounds. Several studies have also shown the conversion of these precursors and metabolites to the corresponding β-carbolines (THBC, 2MTHBC, HTHBC and MTHBC), occurring either through condensation with formaldehyde or through a common intermediate, also occurring during demethylation (Barker *et al*., 1980, 1981a). THBC and MTHBC have been reported as endogenous substances appearing in adrenal and pineal glands, as well as other tissues (Shoemaker *et al*., 1978; Barker, 1982; Barker *et al*., 1984, 1979, 1981b; Kari *et al*., 1983; Beaton and Morris, 1984). All of these precursors and products, except

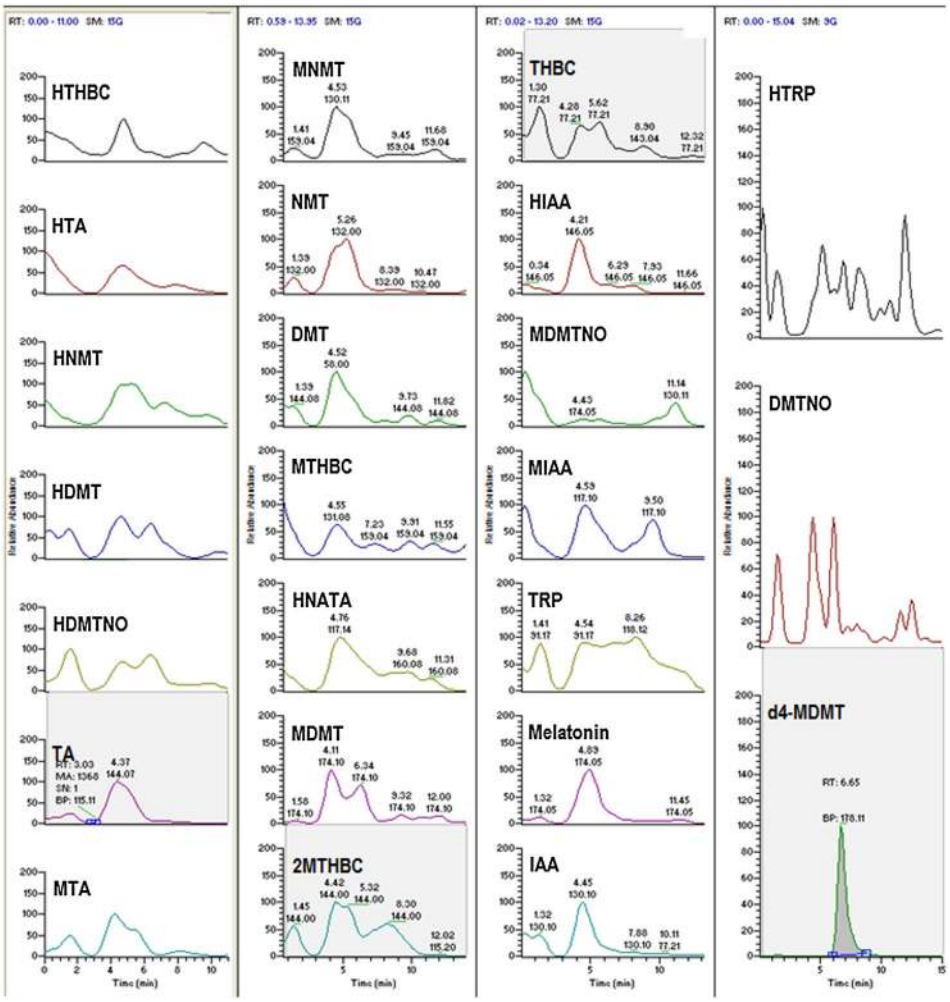

Figure 2A. (A) Chromatogram of artificial CSF (aCSF) fortified with internal standard only (d4-MDMT). (B) Chromatogram of analytes fortified into aCSF. (C) Multi-reaction monitoring ions selected for the detection and confirmation of compounds found in pineal gland CSF microdialysate illustrating retention times and ion ratios compared with reference standards.

the β-carbolines, are substrates for monoamine oxidase (MAO), yielding as a product the respective indoleacetic acids, IAA, HIAA and MIAA. Metabolism of the corresponding primary, secondary or tertiary amines by MAO is the predominant pathway for their degradation. However, the β-carbolines have been shown to possess MAO inhibition activity.

Another major pathway for metabolism of the DMTs is formation of the corresponding *N*-oxides (DMTNO, HDMTNO and MDMTNO). Inhibition of MAO *in vivo* greatly elevates the concentration of the *N*-oxides relative to the IAAs in the metabolism of the DMTs, often making them the major metabolites (Sitaram *et al*., 1987a–c; Sitaram and McLeod, 1990; Kärkkäinen *et al*., 2005; McIlhenny *et al*., 2011, 2012; Riba *et al*., 2012). The *N*-oxides are not substrates for monoamine oxidase (Barker *et al*., 1980, 1981a) and are excreted unchanged in urine (Sitaram *et al*., 1987a–c).

Kynurenine metabolites of tryptophan or the corresponding metabolites of the other indolamines were not included in this assay. Recent data generated *in vivo* suggest that this pathway is not relevant to the metabolism of DMT, and this is also anticipated to be the case for the other DMTs (McIlhenny *et al*., 2011, 2012). Since the analytical study in this case was based on dialysate obtained from the pineal gland, we also monitored the presence of melatonin, HTRP and HNATA.

Ion profiles and monitoring

The mass spectrometer (Quantum Access) settings required to attain the best response and fragment ion information for each compound analyzed are given in Table 1 and are typical for such compounds on this type of instrument. Three compounds gave at least two diagnostic ions while most of the compounds produced at least three ions for monitoring. Three of the compounds analyzed gave four ions, in addition to the acknowledged source of the ions being the monitored protonated molecular weight ion $[(M+1)^+]$. These protonated molecular ions, their product ions and their ratios were used to assist in the identification and confirmation of the analytes. It should be noted that DMT typically gives only two ions, 58^+ and 144^+ (Table 1) under previously reported conditions, as observed by us and others (Kärkkäinen *et al*., 2005; McIlhenny *et al*., 2011, 2012). However, to assist in the confirmation, special conditions for fragmentation were established for the analysis, with DMT giving four diagnostic ions, 58^+, 115^+, 143^+ and 144^+ *m/z* (Table 1 and Fig. 3). The use of more fragment

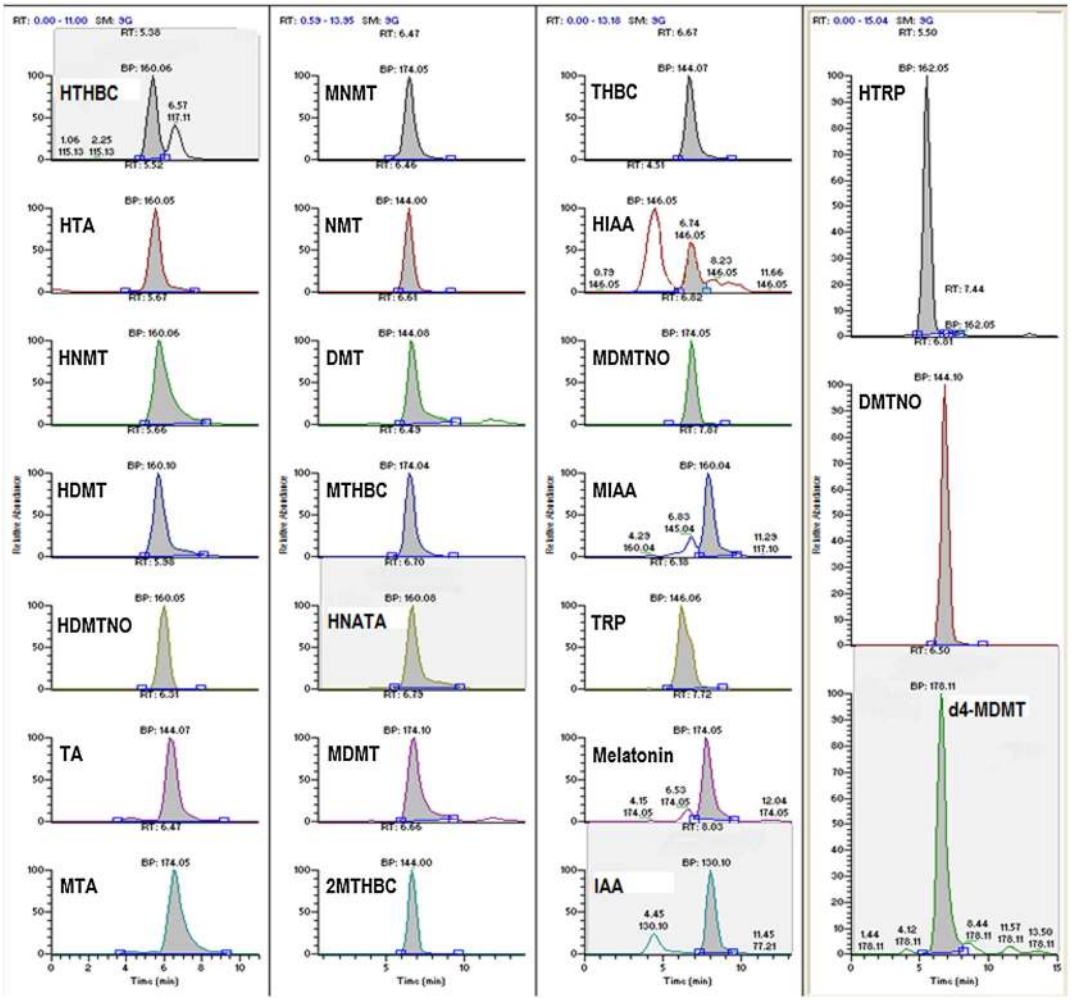

Figure 2B. (Continued)

ions was deemed necessary since the identification of DMT has been controversial (Barker *et al.*, 2012) and low mass ions such as 58^{+} (*m/z*) are not always truly diagnostic.

Chromatography

Average retention time data for the analysis are shown in Table 1. Representative chromatographic data for blank aCSF and analyte-fortified aCSF (10 ng/mL shown), demonstrating the temporal elution of the target analytes, using a representative ion selected for detection for each compound, are shown in Fig. 2 (A and B), respectively. Representative results for compounds detected in pineal microdialysate samples, illustrating the diagnostic ions, their ratios and retention times relative to reference standards, are shown in Fig. 2(C).

The mobile phase gradient developed afforded the ability to allow salts, which are in millimolar concentrations in aCSF, to be eluted to waste before actual ion profile monitoring began, and they were diverted in the first 1.3 min of the analyses. The use of the waste diversion valve assists in keeping the cone and ion source clean. This approach is common practice in LC/MS/MS analysis but was, in this case, also based on other published studies monitoring neurotransmitters and related compounds in rat aCSF microdialysates (Greco *et al.*, 2013; Uutela *et al.*, 2009). The LC gradient, starting with a high water content (98% water–2% acetonitrile), allowed the target analytes to be sufficiently retained at the head of the column to permit the elution of salts to occur. As would be expected, the acidic media of the mobile phase lessened solution ionization of some of the organic acids being analyzed and produced longer retention times for some of them relative to that observed for some of the bases. The acids, bases and amphoterics represented by these 23 compounds were eluted in a 5–9 min window in an order relatively consistent with their polarities in the solvent gradient used. This afforded temporal separation of most of the components of the analysis. All of the compounds were resolved by the combination of time and mass.

The mobile phase was varied during the experiments to move the compounds to shorter (more initial acetonitrile) or longer (higher initial water content for a longer period) retention times and to establish optimum conditions. Pineal dialysate samples shown to be positive for some of the target analytes were treated to demonstrate whether or not the retention time match persisted. In every case the shift in retention times was matched by the detected analyte (data not shown). A more organic initial condition/mobile phase gradient (90:10, water–acetonitrile) was subsequently used for the further confirmation of the analytes using the Exactive OrbiTrap mass spectrometer, creating different (shorter) retention times for the analytes as confirmed from reference standards. Matching of retention times in varying LC conditions is an additional technique to assist in confirmation of unknowns.

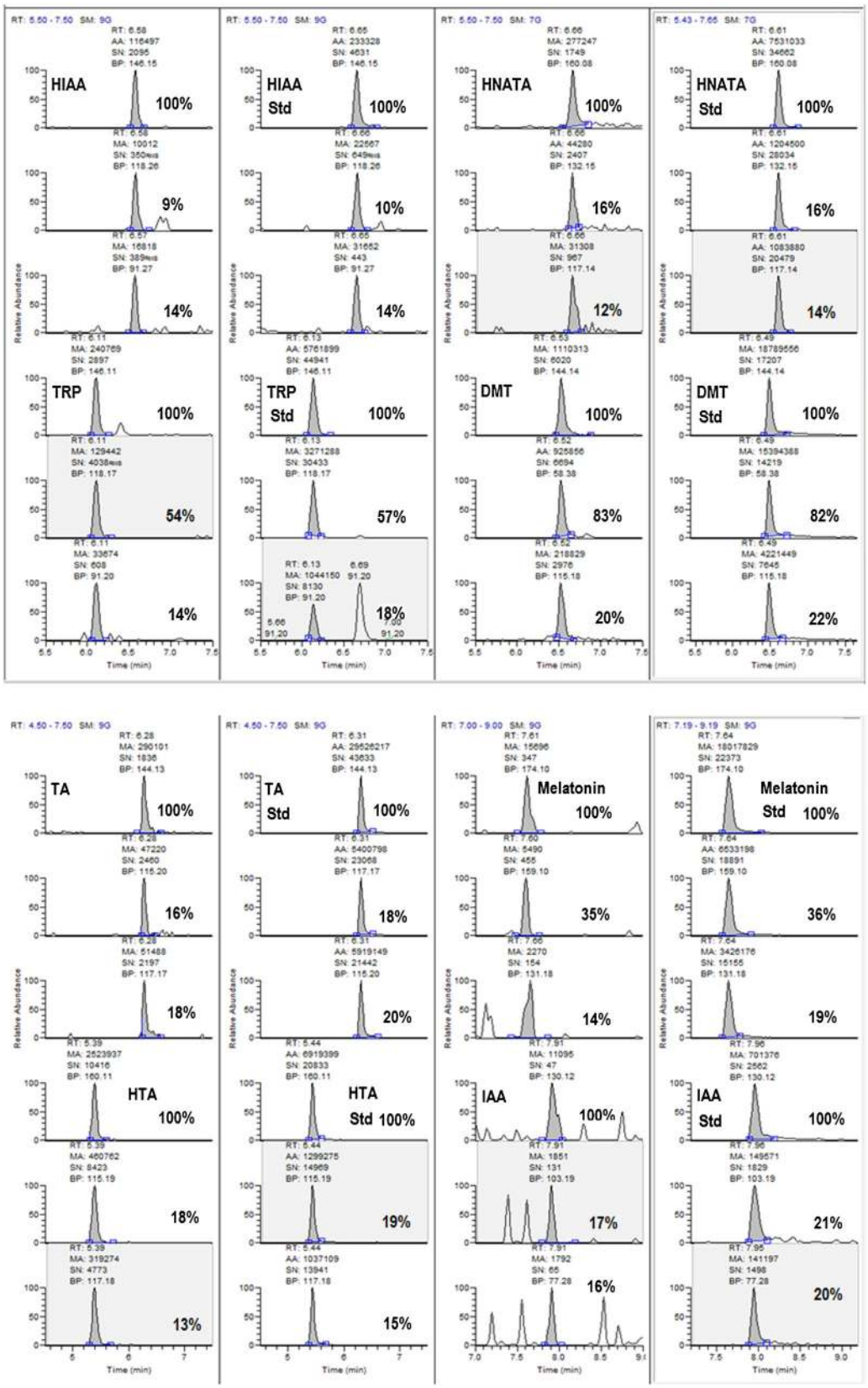

Figure 2C. (Continued)

Selectivity and specificity

An examination of aCSF showed no interfering substances that led to misidentification or false detection (as established by the stated criteria) of any of the target analytes. Pineal dialysate aCSF from rats did show the presence of several of the target analytes as endogenous substances, but did not show any detectable or significant interferences with these or other analytes. Cross-talk between targeted analytes was also examined, since (1) HNMT and MTA, (2) TRP, HDMT, DMTNO and MNMT, (3) DMT and HTHBC, (4) MDMT and HNATA, and (5) HDMTNO and HTRP have, respectively, the same molecular formula and, thus, the same exact mass and protonated molecular ion. However, there was adequate temporal separation of the compounds and differences in mass and mass fragment ion formation such that no detectable cross-talk/interference for any of the compounds was observed. The method as described and performed here suggests that it possesses a high degree of selectivity and specificity.

Analyte stability

All of the compounds examined were stable in solution (artificial CSF) when stored at −80 °C for up to 6 months (longest time measured) and stable at 4 °C for the same period of time. All of the compounds were stable at ambient temperatures for at least 1 week in the dark.

Limits of detection

Limits of detection (LODs) ranged from 0.02 to 2.0 ng/mL (HIAA; Table 1). Standards were analyzed between the range of concentrations of 0.01–25 ng/mL to establish the LODs. The limiting factor in each was the ability to observe the weakest of the established diagnostic ions at a concentration giving a response greater than 3 times baseline noise. The resulting data suggest that the assay is capable of providing reliable analytical results for these compounds with a high degree of sensitivity in this matrix.

Detection, and especially quantitation, of these compounds in tissue microdialysates is complicated by the dynamic aspects of the microdialysis process itself (Chefer *et al*., 2009). Recovery of the compounds to be analyzed, often called the extraction fraction, relative recovery or probe efficiency, can be affected by the type of probe, probe membrane, flow rate, tissue resistance and a number of other factors. For example, the analyses using the system applied in this case showed that the *in vitro* rate of probe recovery was 14% for 5-HT, 15% for *N*-acetylserotonin and 12% for melatonin under our experimental conditions (data not shown). This is in line with other published studies. Several studies have also shown that IAA and HIAA, two of the other compounds detected, are also recovered in this same percentage range using a variety of techniques (Kendrick, 1989). It may also be assumed that the rate of *in vitro* DMT and TA recovery, the last two compounds

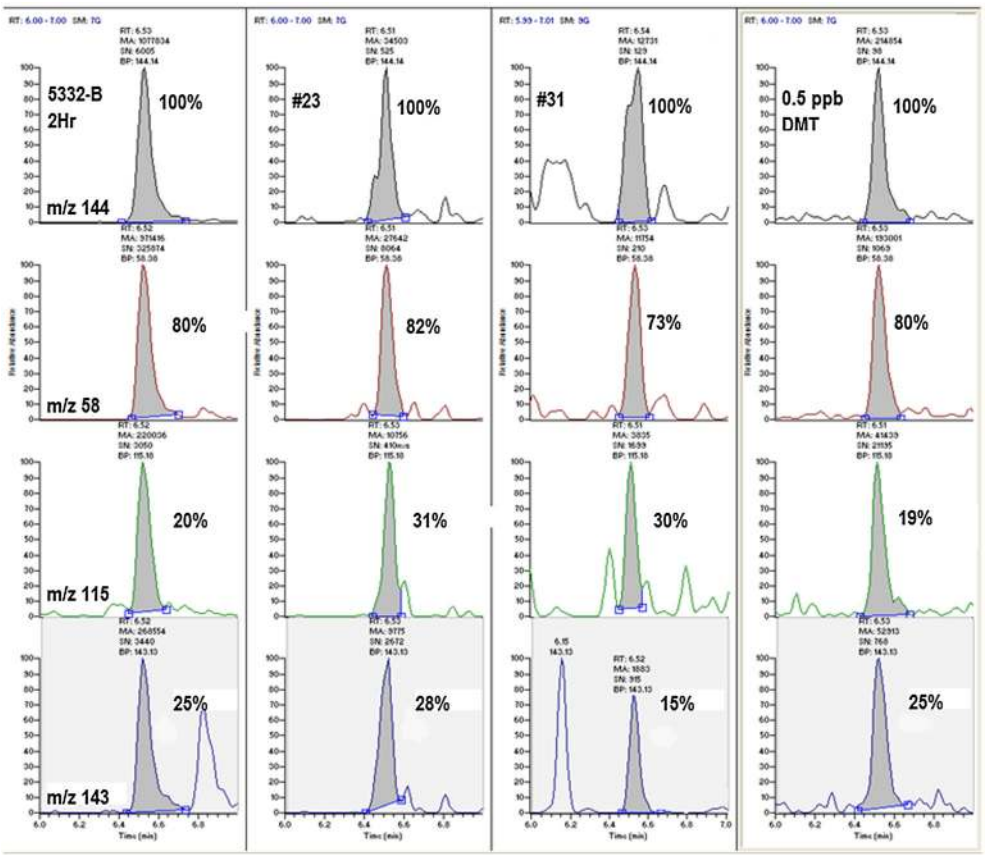

Figure 3. The detection of *N*,*N*-dimethyltryptamine (DMT) in samples 5332-B 2Hr, nos 23 and 31 as determined by retention time, occurrence of all four diagnostic ions and the correct ion ratios (±25% relative) vs a DMT reference standard spiked into aCSF at 0.5 ng/mL (ppb).

detected, would be within this range as well. Given the chemistry of the remaining compounds examined, it is also reasonable to assume that their recovery rate is in the same range, with many being quite water-soluble or closely related in physicochemical properties to the compounds detected. As with any assay, the combination of recovery rate, inherent in microdialysis techniques, and lack of adequate sensitivity may combine to give a negative result.

The present protocol was established for the qualitative analysis or screening of pineal microdialysate and, thus, was not examined for matrix effects. While direct analyses as described here provide a high degree of efficiency, they can also lead to problems arising from the effects of co-eluting matrix components. Such effects can enhance or suppress detectability. This is a particular problem for quantitative analyses. Previous quantitative studies analyzing rat aCSF dialysate for neurotransmitters and related compounds have examined their methodology for matrix effects. For example, Cannazza *et al*. (2012) found no evidence for matrix effects in their assay and concluded that this was due to the fact that an initial high water content mobile phase and use of a diverter valve effectively eliminated inorganic salts that often are the source of ion suppression. Potential interference from proteins is also greatly reduced in microdialysis samples, since the pineal dialysate, in this case specifically, had to pass a dialysis probe with a molecular weight cut-off of 13 kDa. We suggest that future efforts directed toward quantifying the compounds examined here initially screen samples using the method described to first determine which of the many analytes can be confirmed to be present. A suitable method may then be developed that targets these analytes and, thus, simplify the analysis. At that juncture, appropriate validation, including determination of probe recovery rates and matrix effects, should be conducted.

Method performance

The analytical procedure presented here is straightforward, requiring, in our hands, no filtration or dilution of the dialysate for analysis. Such direct analysis limits the ability to gain sensitivity through the process of extraction and concentration. Nonetheless, it avoids losses of some of the compounds vs others in attempting to establish a comprehensive extraction protocol for such a large number of chemically diverse compounds. Further, we are typically dealing with a rather small sample size (240 μL as performed here), which also complicates the use of complex extraction procedures. Such an approach seems ideal for this matrix. We noted, however, that the use of a heated electrospray probe caused significant loss of signal and response in the progression of samples. This problem was remedied by using an ambient temperature setting on the probe and early solvent diversion to waste.

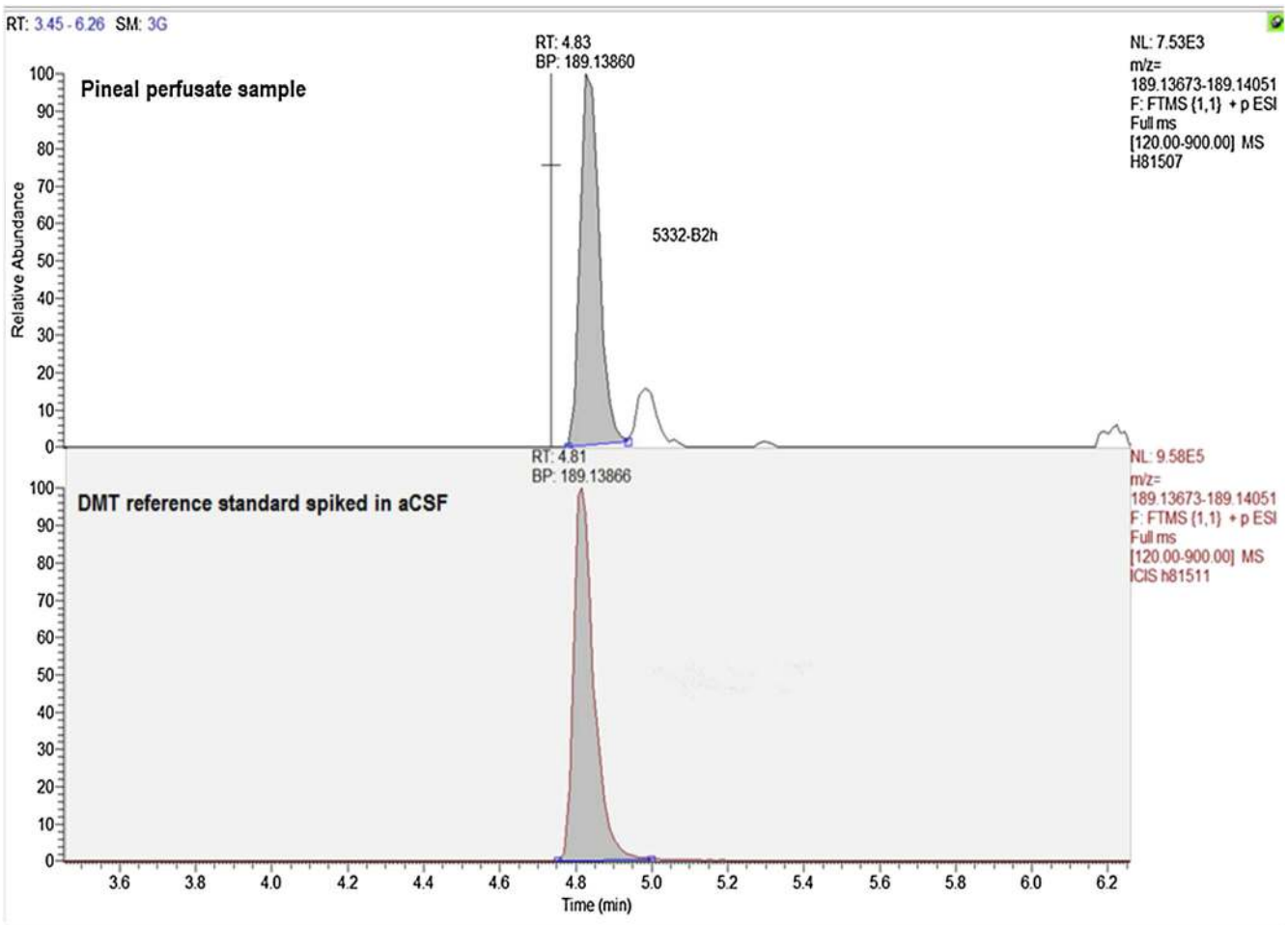

Figure 4. Representative exact mass data for a DMT reference standard and the presumptive DMT peak observed in pineal gland perfusate.

Analytes detected and not detected in rat CSF

Figure 2(C) is illustrative of chromatograms of pineal microdialysate samples, showing the major ions used for monitoring and confirming the analytes. The chromatograms for the analysis of aCSF collected from pineal perfusion (Fig. 2C) show the presence of the compounds HTA, melatonin, TRP, HNATA, HIAA, IAA, TA and DMT. Not all of the compounds were detected at all times in all samples. However, as expected, melatonin and HTA were consistently detected in pineal dialysate samples. It is important to note that samples positive for DMT were also positive for its biosynthetic precursor TA and its terminal metabolite IAA. This data illustrates the need to monitor precursors and metabolites and the need to follow the affected pathways of biosynthesis and degradation, especially with changing conditions in experimentation.

Three additional representative chromatograms and ion traces of samples collected by pineal gland perfusion of freely moving rats confirming the presence of DMT are shown in Fig. 3 and are compared with a reference standard for DMT. Figure 3 illustrates the matches obtained for the retention times, presence of four diagnostic ions and the matches for their ion ratios, all meeting stated criteria for confirmation.

Within the limits of the assay as described and for the small number of samples examined here, there was no indication of the presence of the other compounds analyzed. However, the failure to detect many of these substances should not be taken as evidence that they are not biosynthesized or present in the pineal gland of the rat. In the present study, samples were only collected from pineal gland for 2 h during day time. Longitudinal studies or studies altering physiological conditions may show different data. Nonetheless, despite the presence of the precursor for their synthesis (HTA), no MTA, MNMT, MDMT, MDMTNO or MIAA nor the corresponding β-carboline (MTHBC; pinoline) was detected. Similarly, no HNMT, HDMT, HDMTNO or HTHBC was detected, despite the presence of HTA. However, HIAA was detected in most samples. Also, although TA was detected in some samples as well as DMT, no THBC, NMT, 2MTHBC or DMTNO was observed, although IAA, the terminal metabolite for DMT, TA and NMT, was detected in most samples. Pharmacological intervention may alter these patterns.

Exact mass confirmation of DMT and other detected compounds in rat CSF

Additional confirmation of the identity of detected analytes was obtained using the Thermo Exactive mass spectrometer. Thus, melatonin (calculated exact mass 233.12828 amu/found 233.12849 amu), HTA (177.10224/177.10214 amu), TA (161.10732/161.10735 amu), IAA (176.07061/176.07057 amu), HIAA (192.06552/192.06511 amu), TRP (205.09715/205.09711 amu) and HNATA (219.11280/219.11296 amu) detected in samples were further confirmed, with their exact masses agreeing to within ±0.1–0.9 ppm and matching the retention time for the corresponding reference standards (data not shown) using a modified LC program.

Figure 4 shows representative data obtained for the exact mass measurement of a DMT standard and DMT peaks in rat pineal microdialysate samples using the Thermo Exactive instrument and a different LC program from that used in the screening of samples using the Thermo Quantum Access instrument. Analysis of samples for DMT by exact mass gave 189.13860 amu $[(M+1)^+]$ compared with the calculated exact mass of 189.13866 amu obtained for a DMT reference standard, occurring at the matching retention time for DMT (Fig. 4).

Taken together, the retention time matches (using two different LC methods), the presence of four ions generated by monitoring and fragmenting a molecule with a nominal protonated molecular ion (m/z) of 189^+ and the high-resolution matching of the exact mass for this compound vs a reference standard are all scientifically compelling evidence for the presence of DMT in rat pineal gland microdialysate. As noted, DMT was not detected in all samples, as melatonin and HTA were, and it is possible that the presence of measurable levels of DMT may be subject to yet undetermined physiological conditions or changes as well as time.

DMT has previously been reported in human CSF collected by lumbar puncture (Corbett *et al.*, 1978; Smythies *et al.*, 1979). Its origin in this matrix remains unknown. DMT has also previously been reported to be present in whole rat brain. In 2005, using a sensitive LC/MS/MS approach, Kärkkäinen *et al.* (2005) reported the presence of DMT at low pg/g levels (10 and 15 pg/g) in two whole rat brain samples analyzed from MAO-inhibitor treated animals. In 1984 Beaton and Morris (1984), using GC/MS methodology, also reported DMT as being present in whole rat brain in low ng/g concentrations. This latter study illustrated changing concentrations of DMT in whole brain with age of the animal and also reported the detection of MDMT, THBC and TA. Thus, the present research may be seen as corroborating these studies with regard to DMT but is the first such study to report the presence of DMT in pineal gland and in live animals.

Conclusions

The method described is a simple, sensitive, specific and direct approach to the qualitative analysis of compounds in the tryptophan pathway related to the biosynthesis and metabolism of the endogenous hallucinogens, or DMTs. The method involves the direct injection of rat pineal gland aCSF microdialysate and analysis by LC/MS/MS, using stringent MS confirmatory criteria, including exact mass measurements. It is anticipated that the same approach would also be viable for application to other tissue perfusates, biological fluids or samples with little or no modification. The excellent limits of detection, as well as the capacity to perform qualitative analyses for a large number of compounds in the tryptophan pathway simultaneously, without requiring extraction, make it a valuable research tool, particularly for examining the endogenous hallucinogen pathways in the CNS. We report here for the first time the presence of DMT in pineal gland dialysate obtained from the rat. However, the method will permit the conduct of a range of future experiments, such as measuring possible circadian changes or performing dialysis studies in other brain tissues or in the ventricles. Regardless of the sample source, it is also important to be able to assess the effects of pharmacological treatments, such as MAO inhibition or other physiological interventions, on changes in the presence or concentrations of DMT and the other endogenous hallucinogens, their precursors and/or metabolites.

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