Quantification of endogenous neurotransmitters and related compounds by liquid chromatography coupled to tandem mass spectrometry

Eulàlia Olesti[1,2]*, Jose Rodríguez-Morató[1,2,3]*, Àlex Gomez-Gomez[1,2], Johannes G. Ramaekers[4], Rafael de la Torre[1,2], Óscar J. Pozo[1§].

[1] Integrative Pharmacology & Systems Neuroscience Group, IMIM, Hospital del Mar, Doctor Aiguader 88, 08003, Barcelona, Spain.

[2] Department of Experimental & Health Sciences, Universitat Pompeu Fabra (CEXS-UPF), Doctor Aiguader 88, 08003, Barcelona, Spain.

[3] CIBER de Fisiopatología de la Obesidad y Nutrición (CIBEROBN, CB06/03/028), 15706 Santiago de Compostela, Spain.

[4] Experimental Psychopharmacology Unit, Department of Neurocognition, Faculty of Psychology, Maastricht University, Maastricht, the Netherlands.

*Both authors contributed equally to the manuscript.

[§]Correspondence and request for materials should be addressed to: Óscar J Pozo (opozo@imim.es) Address: c/ Doctor Aiguader 88, 08003, Barcelona, Spain.

Abstract

Neurotransmitters are signaling molecules, playing key roles in neuronal communications in the brain. Drug induced changes in neurotransmitters and other brain metabolite concentration may be used to characterize drugs according to their targeted metabolomics profile. Here, we report the development and validation of a straightforward liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the simultaneous quantification of 16 endogenous small polar compounds in rat plasma and brain homogenates. The method enables the quantification of the neurotransmitters γ-aminobutyric acid, glutamate, acetylcholine and adenosine, as well as choline, glutamine, acetylcarnitine, carnitine, creatine, creatinine, valine, leucine, isoleucine, phenylalanine, tyrosine and tryptophan. After optimizing the sample preparation, chromatographic and spectrometric conditions, the method was successfully validated using the standard addition approach and a hydrophilic interaction chromatography (HILIC) with an amide column. The method was shown to be linear ($r > 0.99$) as all the compounds were within the ± 25% values of intra and inter-day precision and accuracy acceptance. A matrix effect was corrected with the use of 10 isotopically labelled internal standards and the compound stability was evaluated for all compounds. Relevant exaltation of choline (in plasma) and creatinine (in brain) were solved with -20ºC conditions. The applicability of the method was tested by evaluating brain alterations in the concentrations of neurotransmitters and related compounds after the administration of two psychostimulant drugs of abuse (cocaine and methylenedioxypyrovalerone) to rats. A neuro-metabolic fingerprint of each drug was obtained that reflected their pharmacological profile. Altogether, this methodology presents a valuable targeted metabolomics tool for basic and clinical research studies.

Keywords

Liquid chromatography coupled to mass spectrometry, Hydrophilic interaction chromatography, Quantitative method, Brain neurotransmitters, Standard addition, Psychoactive Substances.

Abbreviations

γ-Aminobutyric acid, GABA; acetylcarnitine, ACar; acetylcholine, ACh; adenosine, Ade; carnitine, Car; choline, Ch; cocaine, COCA, creatine, Cr; creatinine, Creat; glutamate, Glu; glutamine, Gln; hydrophilic interaction chromatography, HILIC; isoleucine, Ile; liquid chromatography coupled to mass spectrometry, LC-MS/MS, leucine, Leu; methylenedioxypyrovalerone, MDPV; phenylalanine, Phe; tryptophan, Trp; tyrosine,

Tyr; valine, Val; prefrontal cortex, PFC; cerebellum, Cer; hippocampus, HC;striatum, St; new psychoactive substances, NPS; neurotransmitter, NT.

1. Introduction

Neurotransmitters are endogenous molecules that play an essential role in transmitting signals from a neuron to a target cell across a chemical synapse. Their main function is to communicate information throughout the brain and the rest of the body [1]. General classes of neurotransmitters include monoamines (e.g. serotonin, dopamine, noradrenaline, adrenaline), amino acids (e.g. aspartate, glutamate, glycine, γ-aminobutyric acid), purines (e.g. adenosine, ATP), choline (e.g. acetylcholine), as well as peptides (e.g. neuropeptide Y) and gases (e.g. NO) [2]. The accurate measurement of brain neurotransmitters concentrations (as well as their precursors and metabolites) is a key tool to understand brain function.

To date, a number of methods for the analysis of polar neurotransmitters (e.g. glutamate or γ-aminobutyric acid) and related compounds in biological matrices have been developed. These are mainly based on a separation technique (high-performance liquid chromatography, gas chromatography or capillary electrophoresis) [3,4] coupled to different detection techniques (ultraviolet absorbance, fluorescence, electrochemical, or mass spectrometry) [4–10]. Amongst the previous analytical tools, liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) allows the simultaneous measurement of multiple analytes with high sensitivity and selectivity in a wide dynamic range [11]. Moreover, it presents the possibility of avoiding derivatization agents (largely needed with gas chromatography) and shorting the running-time per sample.

The major challenges for the LC-MS/MS analysis of very polar/ionic endogenous compounds lie on their high hydrophilicity, which cause low recoveries by typical extraction methods [12,13]. In literature, different LC-MS/MS methodologies used distinct approaches to quantify amino acid related compounds. Strategies such as microextractions (liquid-liquid [13] or solid-liquid [12]) with evaporation steps [8,9] have been used for cleaning up and/or pre-concentrating monoamine and amino acid related neurotransmitters. In all cases, these approaches take time and consumables and low recoveries are usually associated to their extractions steps. Alternatively, methods employing a “dilute-and-shoot strategy” would provide a more economical and easier sample preparation by the omission extractions[14]. However, this type of approach is lacking for polar/ionic neurotransmitters.

Recently, we reported a “dilute-and-shoot” approach for the study of tryptophan and tyrosine metabolism including 16 analytes related to serotonin, dopamine and the kynurenine pathway [5]. However, the inclusion of highly polar amino acids neurotransmitters and related compounds such as choline, carnitine or γ-aminobutyric acid was discarded due to lack of retention of these compounds in a reverse phase column (C18). That is common for polar/ionic neurotransmitters which often elute close to the column hold-up volume [15]. Several methodologies have been reported to overcome this lack of retention such as the use of derivatization agents [12,16], modified stationary phases like pentafluorophenyl (PFP) [8] or the hydrophilic interaction chromatography (HILIC) columns [13,17,18]. These methods allowed for the determination of glutamate, glutamine and γ-aminobutyric acid, among other monoamine-related neurotransmitters [8,9,12,13,18,19] but they do not quantify ionic compounds such as cholines, carnitines or creatinines.

Another challenge for the quantification of endogenous compounds is their natural occurrence in biological matrices, which complicates their determination and the validation of the analytical methodology due to the difficulty in obtaining either a matrix-free of analytes or samples with known concentrations of analytes [20,21].

Alterations in the endogenous concentrations of brain neurotransmitters have been described in several diseases [22] and after the administration of drugs of abuse [23]. Thus, targeted metabolomics approaches based on the quantification of neurotransmitters related pathways are potentially useful in a broad number of application fields. An example is the characterization of the mechanism of action of drugs of abuse.

Lately, new emerging substances (commonly called as new psychoactive substances (NPS)) become popular in the drug-market scene [24]. These substances were designed to mimic classical drugs of abuse (such as ecstasy or cocaine) but circumventing law enforcement [25]. Severe adverse effects have been reported for users of these substances [26]. One example of NPS was methylenedioxypyrovalerone (MDPV), a synthetic cathinone described to produce a cocaine-like effect [27,28]. With regard to the pharmacology of this substance, there is an information gap on the brain alterations after the drug administration. The simultaneous quantification of multi-endogenous metabolites could provide a better understanding of the mechanism of action of this novel drugs [29].

Thus, the first objective of this study was to develop and validate a straightforward LC-MS/MS method for the simultaneous quantification of 4 neurotransmitters (γ-aminobutyric acid, glutamate, acetylcholine and adenosine), as well as 12 polar related compounds (choline, glutamine, acetylcarnitine, carnitine, creatine, creatinine, valine, leucine, isoleucine, phenylalanine, tyrosine and tryptophan) (see **Figure 1**) in rat plasma and rat brain homogenates. In the present report we have applied the method of standard addition for the validation procedure. The second objective of this report was to demonstrate the application of the method by evaluating how different psychostimulants [cocaine (COCA) and methylenedioxypyrovalerone (MDPV)] administered to rats alter, in specific brain areas, the concentrations of some of these neurotransmitters and related compounds.

2. Materials and methods

2.1. Chemicals and reagents

Valine (Val), leucine (Leu), isoleucine (Ile), phenylalanine (Phe), tryptophan (Trp), tyrosine (Tyr) and ammonium formate (HPLC grate) were obtained from Sigma-Aldrich (St. Louis, MO, USA). γ-aminobutyric acid (GABA), choline (Ch), creatinine (Creat), creatine (Cr), acetylcholine (ACh), glutamine (Gln), glutamate (Glu), carnitine (Car), acetylcarnitine (ACar) and adenosine (Ade) were obtained from Sigma–Aldrich (St. Louis, MO, USA). All internal standards used were from Toronto Research Chemicals, TRC (Toronto, Canada): GABA-2,2,3,3,4,4-d_6, choline-1,1,2,2-d_4, creatinine-(methyl-d_3), 1-^{13}C-α-^{15}N-glutamine, glutamate-2,3,3,4,4-d_5, phenylalanine-(phenyl-d_5), tyrosine(phenyl-d_4), tryptophan-(indole-d_5), (ribose-$^{13}C_5$)-adenosine, and acetylcholine-(*N*,*N*,*N*-trimethyl-d_9). Cocaine (COCA) was obtained from Sigma-Aldrich (Saint Louis, MO, USA) and methylenedioxypyrovalerone (MDPV) was purchased LGC Standards (Lancashire, UK). Formic acid (98%) and acetonitrile were purchased from Merck (Darmstadt, Germany) and ultrapure water (with a Milli-Q purification system was obtained from Millipore Ibérica (Barcelona, Spain).

2.2. Standards and solutions

Stock solutions of all compounds (at 1 mg/mL) were prepared in a mixture 0.1% aqueous formic acid:acetonitrile (1:2) from solid powdered standards. Working solutions were prepared by diluting stock solutions with the mixture (aqueous formic acid 0.1%: acetonitrile; 1:2) in a concentration range from 1 to 20,000 ng/mL. These working solutions were used for all validation irrespective of the matrix assayed.

2.3 Biological samples

Thirty-six male Wistar Rats, aged approx. 7 weeks and weighing between 200-225 g, were purchased from Charles River. Rats were housed in ventilated cages with controlled temperature (22 ± 1ºC), humidity (55 ± 10%) and under a 12 h light/dark cycle (on at 8 a.m. and off at 8 p.m.). Food and water were available *ad libitum* and animals were handled and habituated to the experimental room one week prior to testing. All animal procedures were conducted in accordance with the standard ethical guidelines (European Communities Directive 86/60-EEC) and approved by the local ethical committee (Comitè Ètic d'Experimentació Animal of the Barcelona Biomedical Research Park; CEEA-PRBB).

To obtain the biological samples, rats were anesthetized with isoflurane and blood was collected by an intra-cardiac puncture and euthanized immediately. Brain was extracted and prefrontal cortex (PFC), cerebellum (Cer), hippocampus (HC) and striatum (St) were freshly dissected on ice bath condition, weighted, placed in liquid nitrogen, and stored at -20ºC until processing. Blood was kept at 4ºC during the experimental collection in an EDTA tube (6 mL) and plasma was obtained by centrifugation of the tube at 4ºC and 1700 g for 15 min.

2.4. Liquid chromatography and mass spectrometry conditions

Quantification of polar neurotransmitters and related compounds in rat plasma and brain homogenates was performed using an Acquity UPLC system (Waters Associates) for the chromatographic separation coupled to a triple quadrupole (Xevo TQ-S micro) mass spectrometer provided with an orthogonal Z-spray-electrospray interface (ESI) (Waters Associates, Milford, MA, USA). The drying and nebulizing gas was nitrogen. The desolvation gas flow was set to 1200 L/h and the cone gas flow to 50 L/h. A capillary voltage of 3 kV was used in positive ionization mode. The nitrogen desolvation temperature was set to 600ºC and the source temperature to 150ºC. Collision gas was argon and the injection volume was 5 μL.

The chromatographic separation was achieved at 55ºC using an ACQUITY UPLC BEH Amide 1.7 micron (2.1 x 100 mm) (Waters Associates), at a flow rate of 600 μL/min. Mobile phase A was ammonium formate 25 mM with formic acid (0.01% v/v) dissolved in a mixture acetonitrile:water (9:1). Mobile phase B was ammonium formate 25 mM with formic acid (0.01% v/v) in water. A gradient program was used for the separation of the analytes; the percentage of mobile phase B linearly changed as follows: 0 min, 10%; 0.5 min, 10%; 2 min, 40%; 2.5 min, 40%; 2.6 min, 10%; 3.5 min, 10%. Analytes

were determined by a Selected Reaction Monitoring (SRM) method by acquiring two transitions for each compound as specified in **Table 1**. The most specific transition was selected for quantitative purposes. MassLynx software V4.1 and TargetLynx XS were used for data management.

2.5. Sample preparation and extraction

2.5.1. Internal standard mixture

An internal standard mixture containing 500 ng/mL of GABA-d_6, 250 ng/mL of Ch-d_4, 65 ng/mL of Creat-d_3, 250 ng/mL of ^{13}C-^{15}N-Gln, 125 ng/mL of Glu-d_5, 250 ng/mL of Phe-d_5, 250 ng/mL of Tyr-d_4, 250 ng/mL of Trp-d_5, 250 ng/mL of $^{13}C_5$-Ade, and 160 ng/mL of ACh-d_9 was prepared by dissolving the appropriate amounts of the stock solutions in water.

2.5.2. Plasma samples

The quantification of the 16 analytes in rat plasma was performed using two alternative treatment (diluted and undiluted) as follows. For the diluted treatment, an aliquot of 10 μL of plasma was diluted 1:20 with 200 μL of water and then 10 μL were transferred to a new glass tube and spiked with 50 μL of the internal standard mixture. Then, 130 μL of acetonitrile was added to precipitate the proteins. After centrifugation (5 minutes; 4000 rpm; 2650 g) the supernatant was transferred to a microvial and directly injected into the LC-MS/MS system. For the undiluted treatment, an aliquot of 10 μL of plasma was transferred into a glass tube, spiked with 50 μL of the internal standard mixture and treated as described for the diluted treatment. The standards used for calibration were subjected to both procedures.

2.5.3. Brain homogenates

Brain areas (PFC, Cer, HC or St) were homogenized with 1 mL tissue grinder dounce (Wheaton, USA). The tissue was processed in 400 μL of formic acid at 0.1%. Thirty movements “loose”, followed by thirty movements “tight” were used for homogenization. Proteins were precipitated with 800 μL of pure acetonitrile. The mixture was centrifuged (10 min; 4ºC; 15700 g) and the supernatant was kept at -20ºC until analysis.

The quantification of the 16 analytes in rat brain homogenates was performed in the same way that plasma samples with the only difference that a 1:100 dilution (instead of the 1:20) was required for the diluted procedure due to the higher brain concentrations of some of the analytes. To do so, 10 μL of brain homogenate were diluted with 1000

225 μL of water, and 10 μL of the mixture were transferred to a new tube, spiked with 50 μL
226 of the internal standard mixture and treated as previously described.

227 2.6. Method validation

228 The samples selected for validation were analyzed using the described sample
229 treatment and their concentrations were estimated by both the standard addition and
230 the external calibration methods. Linearity, accuracy, precision, lower limit of detection
231 (LOD), lower limit of quantification (LLOQ), matrix effect, and stability were tested as
232 described in the next subheadings.

233 2.6.1. Calibration curves and linearity

234 Calibration standards with a mixture of all the analytes were prepared (as the undiluted
235 samples) in ultrapure water at 12 concentration levels (5, 10, 25, 50, 100, 250, 500,
236 1000, 2500, 5000, 10000 and 20000 ng/mL). Then the calibration range for every
237 specific analyte was selected as specified in **Table 2** according to the expected
238 concentrations in the biological matrices and having at least 6 calibration concentration
239 levels in addition to the blank sample. Calibration curves with their corresponding slope
240 (s), intercept, correlation coefficient (r), and coefficient of determination (r^2) were
241 calculated by weighting (1/x) least squares linear regression of the peak area ratio
242 (analyte/internal standard) *versus* the concentration of the standards using SPSS for
243 Windows, version 25.0. The method was considered linear in the selected range if a
244 correlation of determination (r^2) greater than 0.98 was obtained and if the concentration
245 residuals were ≤ 20%. The quantification of the analytes was calculated by
246 interpolation of the response ratios on the calibration curves. The concentration
247 residuals were calculated as follows:

248 Concentration residuals (%) = (interpolated concentration – nominal
249 concentration)*100/interpolated concentration.

250 2.6.2. Calculation of concentrations using the standard addition method

251 For calculating sample concentrations, each sample of analysis (either brain
252 homogenate or plasma) containing an unknown concentration of endogenous analytes
253 was divided into 15 individual aliquots: three of them were not spiked whereas the
254 remaining twelve were spiked with known and increasing amounts of analytes with the
255 same twelve concentration levels specified in section 2.6.1. In this way, a standard
256 curve could be created for every analyte in exactly the same matrix in which the
257 analysis was performed. A solvent calibration curve was also injected in the same

batch. Two concentrations were obtained: the “calculated concentration” and the “actual concentration”. A visual representation of both is shown in **Supplementary Figure 1**.

Firstly, the “calculated concentration” of the sample was obtained by the interpolation of the response (analyte/internal standard) obtained for the three non-spiked aliquots in the solvent standard curve.

Secondly, for establishing the “actual concentration” a standard curve was generated for every analyte in every tested sample by plotting the response *versus* the added concentration of the standards. Amongst the multiple added concentrations points of this standard addition curve 4 were selected: the corresponding at 0 (average response of the instrument of the three non-spiked samples), the added concentration similar to half of the “calculated concentration” (X/2), the added concentration similar to the “calculated concentration” (X), and the added concentration similar to twice the “calculated concentration” (2X). The rest of the points were discarded for the analysis. Then, a straight line fitting how the instrument response (Y-axis) changes depending on the added concentration of analyte (X-axis) was plotted. Finally, the “actual concentration” in the sample was determined by the extrapolation of the regression line as the negative X-intercept of the calibration curve prepared for that particular analyte.

2.6.3. Intra- and inter-assay accuracy and precision

Intra- and inter-assay accuracy and precision were evaluated by the standard addition approach in 12 different rat brain homogenates and 12 different rat plasmas. Accuracy values for every analyte in each matrix were calculated by dividing the “calculated concentration” by the “actual concentration” (see subheading 2.6.2) and it was expressed as a percentage. The same experiments were performed on 3 different assays to assess inter-assay accuracy and precision. The 12 matrices were analyzed in three different assays (six matrices on the first assay, and three matrices on the second and third assays). Intra-day accuracy was calculated as the average of the 6 individual accuracy values of the 6 matrices analyzed on the first validation assay. Intra-day precision was calculated as the standard error (standard deviation/mean) of the 6 matrices analyzed on the first validation assay. Inter-day accuracy of the method was calculated as the average of the 12 individual accuracy values of the 12 matrices analyzed over the three validation assays. Inter-day precision was calculated as the standard error of the 12 matrices analyzed over the validation assays. The method was considered accurate and precise if accuracies were in the range 80%-120% and standard errors were below 20%.

2.6.4 Limit of detection

The limit of detection (LOD) was calculated from the lowest standard used in the calibration curve. The LOD was estimated as the concentration providing a S/N = 3.

2.6.5 Matrix effect

Because the targeted analytes were endogenous and no blank matrix was available, the estimation of the matrix effect was evaluated by the standard addition approach. As we have used a dilute and shoot procedure, the slopes of post-extraction spiked samples were not available either. Therefore, matrix effect was obtained by comparing the slopes obtained by solvent calibration (n=4) and the slopes of the standard addition samples (n=6). The matrix effect was calculated by applying the following formula:

$$\text{Matrix effect} = [1 - (\text{mean slopes in matrix} / \text{mean slopes in water})] * 100.$$

2.6.6. Stability

The estimation of the stability of the compounds was evaluated by preparing triplicates of: QC (standard concentration + matrix), solvent standard (SS) (standard concentration + ultra-pure water) and blank matrix (BM) (matrix + ultra-pure water). The standard concentration added was 5 times higher the endogenous concentration of the analytes in BM and it was identically added in QC and SS. These samples were kept for 0, 1 and 3 days at room temperature (22ºC), for 0, 1, 3 and 7 days in the fridge (4ºC) and 0, 7 and 14 days in the freezer (-20ºC). The stability was evaluated by comparing the variation of the standard concentration prepared in different days with the fresh analysis (day 0) at the same storage conditions. The standards concentrations values were expressed as a percentage with the following formula: (QC - BM)/SS. All samples were analyzed in the same analytical batch.

The stability of the targeted analytes after freeze-thaw cycles was also evaluated in both matrices (plasma and brain). Each sample (n=6) was distributed in 4 aliquots. One aliquot was directly analyzed and taken as reference value. The other 3 were subjected to 1, 2, and 3 freeze-thaw cycles respectively. Freeze-thaw stability was determined by comparing the values of the different aliquots with the reference value.

2.7. Method application

2.7.1. Study design

For the proof of concept, 18 male Wistar rats were assigned to 3 different treatments (n=6/group) as follows: saline vehicle (0.9% NaCl), 20 mg/kg of cocaine and 4 mg/kg of

methylenedioxypyrovalerone (MDPV). All treatments were administered intraperitoneally. One hour after treatment, rats were anesthetized under isoflurane and plasma and samples were obtained as described in subheading 2.3. All groups of rats were sacrificed 1h post drug or vehicle administration.

2.7.2. Statistical analysis/Data treatment

The targeted analytes were normalized with the weight of the brain area and were represented as a ratio value, which was obtained by dividing the analyte concentration after drug or vehicle administration with the median of the analyte concentration after vehicle treatment. This ratio was presented as a percentage and represented as bars-graph. The statistical analysis between the concentrations of the targeted analytes after vehicle or drug administration was performed with the non-parametric U-Mann Whitney test (SPSS, version 21.0). Significant differences were expressed as *$p<0.05$ and **$p<0.01$.

3. Results and discussion

3.1. Method development and optimization

3.1.1. Preparation of stock and working solutions

The solvent used in the preparation of the stock solutions of the analytes and their corresponding internal standards was found to be a key factor that conditioned the linearity of the analytes. The use of 100% methanol resulted in linearity problems for the majority of the analytes, especially at the high concentrations of the calibration curves. Alternatively, the use of 0.1% acid formic (HCOOH) and acetonitrile (1:2) to dissolve the standards was appropriate to overcome these problems. In a similar way, the working solutions were prepared by serial dilution of the stock solutions using a mixture of aqueous 0.1% HCOOH:acetonitrile (1:2).

3.1.2. Sample preparation

A dilute-and-shoot strategy was used for the sample preparation protocol due to its simplicity [14]. The developed method requires a small amount of sample (10 μL) and the sample preparation is very straightforward: after spiking with 50 μL of internal standard mixture (containing 10 isotopically labelled internal standards), the proteins are precipitated using 130 μL of acetonitrile and the sample is directly injected into the LC-MS/MS system. The low volumes required (total amount of 190 μL), together with the low amount of injected (5 μL) allowed carrying out the whole sample preparation

procedure inside of the same glass conical insert used for the analysis. The use of acetonitrile as precipitating agent enables that the acetonitrile used for the protein precipitation could be suitable for the direct injection into the amide column, avoiding the need of any evaporation step. Moreover, the absence of solid-phase extraction or liquid-liquid extraction represents not only a way to reduce sample manipulation but also a way to reduce the analysis time and costs.

3.1.3. Optimization of LC Conditions

Due to the high polarity of the selected analytes, we selected an Acquity UPLC BEH Amide column (2.1x100 mm with 1.7 μm particle size) for the chromatographic separation. Despite the specificity of the MS/MS detector, a suitable chromatographic separation was required in order to separate Glu and Gln which were found to interfere each other since both compounds share the same product ions (m/z 130, m/z 84 and m/z 56) and have precursor ions only differing 1 unit (m/z 148 for Glu and m/z 147 for Gln). In order to reach this separation trying to keep the minimum chromatographic time, several of the variables affecting the chromatography were evaluated and optimized. The addition of 25 mM ammonium formate to both mobile phases resulted in an important improvement in the chromatographic peak shape. In order to facilitate the continuous and homogenous salt content during the chromatographic run, 10% of water was added into the acetonitrile to prepare the organic mobile phase. Under these conditions and at a flow of 0.6 mL/min an appropriate separation was achieved in 3.7 min.

The final organic content of the extract and the injection volume also played a critical role in the method performance. Thus, whereas a high content of acetonitrile was required to obtain a suitable chromatographic peak, small amounts of water were also required to solubilize the analytes. A final content of aqueous:acetonitrile (1:2) was selected as a compromise between solubility of the analytes and chromatographic behavior. Under these conditions a maximum injection volume of 5 μL was found to provide suitable chromatographic peaks.

3.1.4. Optimization of MS/MS Conditions

All analytes have an efficient electrospray ionization behavior as all of them either have easily-ionizable basic nitrogen atoms or are already cationic analytes. This fact made possible the dilute-and-shoot approach using positive ionization mode and detecting the ionized analytes as $[M]^+$ (in the case of the quaternary ammonium compounds) and $[M+H]^+$ (for the rest of the compounds).

Selected reaction monitoring (SRM) transitions were optimized in order to provide the highest specificity and sensitivity. The retention times, together with the two transitions and the optimized collision energy values of each analyte are shown in **Table 1**. It is worth mentioning that, due to their close retention times, specific transitions were selected to distinguish isobaric Leu (132-43) and Ile (132-69), rather than the shared and abundant 132-86 transition corresponding to the loss of HCOOH [5]. In a similar way, choline and GABA were (two compounds sharing the precursor ion 104 and the product ion 45) could be unmistakably differentiated by having a good chromatographic separation and by selecting specific transitions (104-87 for GABA and 104-60 for Ch).

3.2. Method validation

The method was found to be linear for all the analytes as satisfactory determination coefficients (r^2>0.98) were obtained for all of the analytes in the selected ranges (**Table 2**).

The LOD achieved by the method allowed for the quantification of all 16 analytes at endogenous brain concentrations (**Table 2**). In the case of plasma samples, the sensitivity of the method was not enough to determine concentrations of GABA, ACh and Ade which were below the LOD (5 ng/mL) for these analytes in all samples tested (**Table 2**).

Intra- and inter-assay accuracies presented acceptable values for the 16 compounds. In fact, both intra- and inter-assay accuracies were commonly in the range 85-115% (**Table 2**) for all analytes in both matrices with the only exception of the inter-assay value for Val and ACar in plasma (both 84%). The method was also found be precise, with as intra-assays RSD below 15% for all analytes in both matrices with the only exception of Cr in brain (17%). Inter-assay RSDs were commonly below 20% (**Table 2**) with the only exception of Tyr in brain (24%).

The matrix effect for the analytes and their corresponding internal standards assessed by comparing the slopes of external calibration curves *versus* standard addition curves revealed moderate matrix effect ranging from -18% up to 35% in brain homogenates and from -21% up to 45% in plasma (See **Table 2**). Although the most part of the analytes had acceptable matrix effect values (±20%), five of the analytes (Ch, Cr, Ile, Car, and ACar) had and ion enhancement higher than 20% whereas one analyte (Gln) had ion suppression of 21%. The use of labeled internal standards was able to diminish these effects as when the analyte/internal standard ratios were employed the matrix effect values of the previous compounds were acceptable (±20%), evidencing the

importance of using appropriate internal standards for the accurate quantification of these compounds.

Regarding the stability of the targeted compounds, no alterations of the analytes concentrations were found after 3 freeze-thaw cycles either in plasma and brain (see supplementary table 1).Considering the storage conditions, all compounds were unaltered (QC-BM ± 20%) in plasma at -20ºC conditions for a period of two weeks, with moderate losses of ACar, Leu and Ile (modifications of between 30-40% of the QC-BM). At 4ºC, we observed, a substantial exaltation of plasmatic Ch concentrations after 7 days (3-folds the QC-BM). Indeed, at room temperature (22ºC) for 3 days, Ch presented a 5-fold increase in comparison with the fresh analysis day. This increase in Ch concentrations as a function of time is in agreement with previous reports [30]. Therefore, for the analysis of Ch concentrations in plasma a fast freezer procedure was essential for its correct conservation and quantification. Furthermore, we suspect that the protein precipitation (with acetonitrile) could retard the ex-vivo formation of Ch since spiked samples showed a slower increase of Ch. Regarding the rest of analytes in plasma, a slight drop in ACar values was observed at 4ºC after 3 days, but no significant alterations for the rest of compounds.

In brain extracts, all compounds were stable for 14 days at -20ºC. At 4ºC, all compounds were found to be stable for 3 days but after 7 days, amino acid neurotransmitters (GABA, Glu and Gln) presented a 2-fold exaltation, whereas Creat presented an increase of approximately 60%. With regard to the room temperature condition, only Creat was altered (3.5-fold increased) after 3 days at 22ºC in comparison with the fresh day. As described for plasma, a rapid freeze procedure is required for their analysis.

3.3. Method application

To prove the applicability of the developed methodology we measured brain and plasma concentrations of the 16 metabolites in male Wistar rats (n=18; 6 per group) 1 hour after the administration of vehicle (saline solution), MDPV, and COCA in order to evaluate the similarities and discrepancies between the alterations produced in the selected analytes by both drugs of abuse. The validated method was able to quantify 16 metabolites in all rat brain homogenates. All real samples were within the validated concentration range. Characteristic chromatograms obtained from a real sample are shown in **Figure 2**.

Even though these drugs are described to principally alter the monoamine neurotransmitters system [27], both COCA and MDPV induced significant changes on the targeted analytes in brain, at 1h post drug administration, in comparison with the vehicle treatment (see supplementary tables 2-3). These changes can be attributed to the drug administration as they were not observed after vehicle administration.

Overall, the targeted metabolomics profile of MDPV was similar to COCA (with nuances in the intensity of the compounds alterations and with opposite variations in metabolites such as Ade, ACar or ACh). The neuro-metabolic fingerprint of each drug for the analytes determined (other relevant monoamine neurotransmitters and related compounds are not included in the present report) was in accordance with the drug pharmacology described in-vitro, in animals' experiments [27] and with users reporting similar effects for both substances [31].

Several of the detected alterations are in agreement with previously reported variations produced by these drugs. Thus, both psychostimulants produced a strong increment in Creat brain concentrations (around 5-folds, see **Figure 3a**), which was also reported in the urine of an intoxicated consumer of MDPV [32]. Additionally Cr concentrations were 2-fold boosted after COCA injection (**Figure 3a**), which could be related with the incidence of rhabdomyolysis syndrome after COCA ingestion [33,34]. Moreover, an extensive increase in choline concentrations (5-7 fold, see **Figure 3b**) was observed in hippocampus, in agreement with the reduction of the Ch acetyltransferase activity described after COCA administration [35]. Nevertheless, a decrease in ACh concentrations was notably observed after MDPV treatment but boosted values were found after COCA treatment (**Figure 3b**).

Furthermore, COCA also induced a 2.5-fold increase of Car and ACar, whereas MDPV deeply decreased ACar concentrations in comparison with the vehicle administration (**Figure 3c**). Finally, COCA administration also produced a large increase of Ade (3-fold see **Figure 3d**) and MDPV depleted metabolite concentrations (9-fold, see **Figure 3d**). As far as we know, these changes were not reported previously in the literature.

In summary, the developed method is able to properly determine 16 polar neurotransmitters and related compounds at the endogenous concentrations present in plasma and brain. The precision of the method is adequate to detect the changes produced in the metabolites after the intake of several drugs. Compared with previously reported methods, our procedure has shortened 2 to 7 folds the run time analysis[4,6–9,12,13,16,18,19,36] with a straightforward sample procedure (avoiding derivatizations[6,7,16] or extractions methods[4,12,13]). Furthermore, the method is

strengthen with the use of a low sample volume (up to 10 folds in brain tissue[16,19,36] and 3[36] to 30[6] folds in plasma). The use of lower sample volumes is especially important in brain regions analysis where the amount of sample is usually limited. Using low sample volumes facilitates the incorporation of this methodology to a set of brain targeted metabolomics approaches. Regarding the targeted analytes, a wider coverage of neurotransmitters, precursors, metabolites and related compounds is provided (by including comprehensive cholines, creatinines, long neutral amino acids, glutamines neurotransmitters and a purine in a single analysis) in comparison with other studies[4,6–9,12,13,16,18,19]. Finally another relevant output of the validated methodology is that, (un-commonly in other studies [4,6–8,12,13,16]) it is validated in two different matrices: brain homogenates and plasma.

Conclusions

In the present report, we provide an analytical method for the proper measurement of 16 small polar compounds (polar neurotransmitters and related compounds) in rat brain and plasma. Compared with previously reported methods dealing with the determination of neurotransmitters, the presented approach provides several analytical improvements such as (i) lowering sample volume, (ii) lowering the total run time, (iii) straightforward sample preparation, (iv) having a wide analyte coverage and (v) by being validated in 2 different matrices. In all, the high method throughput is ideal for targeted metabolomics approaches in which the accurate measurement of a large number of samples and analytes is required. This fact has been demonstrated by the application of the method to evaluate short-term (1 hour) effects of COCA and MDPV on the selected target metabolome. Specific changes detected are in accordance with their known mechanisms of action. This method represents a valuable tool for the study the mechanism of action of drugs targeting the central nervous system and can also be applied to study alterations in brain neurotransmitters that occur in neurodegenerative and psychiatric diseases. The wide coverage of the presented methodology makes it applicable for many clinical and preclinical studies as the alterations of the studied compounds are present in many metabolic disorders.

Acknowledgements

The authors are very grateful with Cristina Fernández-Avilés and Patricia Robledo for their technical support during the experimental procedures in the animals' facilities.

Funding

This work was supported by the European Commission (Drugs Policy Initiatives, Justice Programme 2014-2020, contract n^{o}. HOME/2014/JDRU/AG/DRUG/7082, Predicting Risk of Emerging Drugs with In silico and Clinical Toxicology).

JRM is acknowledged for the EU's Horizon 2020 research and innovation program under the Marie Sklodowska-Curie grant Nº 712949 (TECNIOspring PLUS) from the Agency for Business Competitiveness of the Government of Catalonia. The Spanish Health National System is also acknowledged for the contract of OJP (CPII16/00027).

Declaration of conflicting interest

The authors declare no potential conflicts of interest.

References

[1] A.Y. Deutch, Neurotransmitters, in: L.R. Squire, D. Berg, F. Bloom, S. Lac, A. Ghosh, N. Spitzer (Eds.), Fundam. Neurosci., San Diego, 2013: pp. 117–38.

[2] J. Cuevas, Neurotransmitters and their Life Cycle, in: xPharm Compr. Pharmacol. Ref., 2007: pp. 1–7.

[3] S. Parrot, V. Sauvinet, J. Xavier, D. Chavagnac, L. Mouly-badina, L. Garcia-larrea, et al., Capillary electrophoresis combined with microdialysis in the human spinal cord : A new tool for monitoring rapid peroperative changes in amino acid neurotransmitters within the dorsal horn, Electrophoresis. 25 (2004) 1511–1517. doi:10.1002/elps.200305852.

[4] J. Yeon, N. Hyun, M. Sook, H. Suk, H. Pyo, J. Hong, Profiling analysis of biogenic amines and their acidic metabolites in mouse brain tissue using gas chromatography – tandem mass spectrometry, J. Chromatogr. B. 940 (2013) 94–103. doi:10.1016/j.jchromb.2013.09.026.

[5] J. Marcos, N. Renau, O. Valverde, G. Aznar-laín, I. Gracia-rubio, M. Gonzalez-sepulveda, et al., Targeting tryptophan and tyrosine metabolism by liquid chromatography tandem mass spectrometry, J. Chromatogr. A. 1434 (2016) 91–101. doi:10.1016/j.chroma.2016.01.023.

[6] L. Zhao, S. Zheng, G. Su, X. Lu, J. Yang, Z. Xiong, et al., In vivo study on the neurotransmitters and their metabolites change in depressive disorder rat plasma by ultra high performance liquid chromatography coupled to tandem mass spectrometry, J. Chromatogr. B. 988 (2015) 59–65. doi:10.1016/j.jchromb.2015.02.029.

[7] S. Nurullah, S.E. Tague, C. Lunte, Analysis of amino acid neurotransmitters from rat and mouse spinal cords by liquid chromatography with fluorescence detection, J. Pharm. Biomed. Anal. 107 (2015) 217–222. doi:10.1016/j.jpba.2014.12.024.

[8] A. Wojnicz, J. Avendaño, A.I. Casas, A.E. Freitas, M.G. López, A. Ruiz-nuño, Simultaneous determination of 8 neurotransmitters and their metabolite levels in rat brain using liquid chromatography in tandem with mass spectrometry : Application to the murine Nrf2 model of depression, Clin. Chim. Acta. 453 (2016) 174–181. doi:10.1016/j.cca.2015.12.023.

[9] F. Huang, J. Li, H. Shi, T. Wang, W. Muhtar, M. Du, et al., Simultaneous quantification of seven hippocampal neurotransmitters in depression mice by LC – MS / MS, J. Neurosci. Methods. 229 (2014) 8–14. doi:10.1016/j.jneumeth.2014.04.004.

[10] S. Parrot, P. Neuzeret, L. Denoroy, A rapid and sensitive method for the analysis of brain monoamine neurotransmitters using ultra-fast liquid chromatography coupled to electrochemical detection, J. Chromatogr. B. 879 (2011) 3871–3878. doi:10.1016/j.jchromb.2011.10.038.

[11] J. Rodríguez-Morató, Ó.J. Pozo, J. Marcos, Targeting human urinary metabolome by LC–MS/MS: a review, Bioanalysis. (2018).

[12] E. Cudjoe, J. Pawliszyn, Optimization of solid phase microextraction coatings for liquid chromatography mass spectrometry determination of neurotransmitters, J. Chromatogr. A. 1341 (2014) 1–7. doi:10.1016/j.chroma.2014.03.035.

[13] L. Konieczna, A. Roszkowska, M. Nied, B. Tomasz, Hydrophilic interaction chromatography combined with dispersive liquid – liquid microextraction as a preconcentration tool for the simultaneous determination of the panel of underivatized neurotransmitters in human urine samples, J. Chromatogr. A. 1431 (2016) 111–121. doi:10.1016/j.chroma.2015.12.062.

[14] K. Deventer, O.J. Pozo, A.G. Verstraete, P. Van Eenoo, Dilute-and-shoot-liquid chromatography-mass spectrometry for urine analysis in doping control and analytical toxicology, TrAC - Trends Anal. Chem. 55 (2014) 1–13. doi:10.1016/j.trac.2013.10.012.

[15] P. Jandera, Stationary and mobile phases in hydrophilic interaction chromatography: a review, Anal. Chim. Acta. 692 (2011) 1–25. doi:10.1016/j.aca.2011.02.047.

[16] X. Zhao, S. Zhu, H. Yang, J. You, F. Song, Simultaneous determination of amino acid and monoamine neurotransmitters in PC12 cells and rats models of Parkinson's disease using a sensitizing derivatization reagent by UHPLC–MS/MS, J. Chromatogr. B. 995-996 (2015) 15–23. doi:10.1016/j.jchromb.2015.05.017.

[17] W. Zhou, B. Zhu, F. Liu, C. Lyu, S. Zhang, C. Yan, et al., A rapid and simple method for the simultaneous determination of four endogenous monoamine neurotransmitters in rat brain using hydrophilic interaction liquid chromatography coupled with atmospheric-pressure chemical ionization tandem mass spectrometry, J. Chromatogr. B. 1002 (2015) 379–386.

[18] A. Forgacsova, J. Galba, R.M. Garruto, P. Majerova, S. Katina, A novel liquid chromatography/mass spectrometry method for determination of neurotransmitters in brain tissue: Application to human tauopathies, J. Chromatogr. B. 1073 (2018) 154–162. doi:10.1016/j.jchromb.2017.12.015.

[19] M.S. Bergh, I. Lise Bogen, E. Lundanes, A.M. Leere Oiestad, Validated methods for determination of neurotransmitters and metabolites in rodent brain tissue and extracellular fluid by reversed phase UHPLC–MS/MS, J. Chromatogr. B. 1028 (2016) 120–129. doi:10.1016/j.jchromb.2016.06.011.

[20] R. Thakare, Y.S. Chhonker, N. Gautam, J.A. Alamoudi, Y. Alnouti, Quantitative analysis of endogenous compounds, J. Pharm. Biomed. Anal. 128 (2016) 426–437. doi:10.1016/j.jpba.2016.06.017.

[21] N.C. Van De Merbel, Quantitative determination of endogenous compounds in biological samples using chromatographic techniques, Trends Anal. Chem. 27 (2008) 924–933. doi:10.1016/j.trac.2008.09.002.

[22] M. Perry, Q. Li, R.T. Kennedy, Review of recent advances in analytical techniques for the determination of neurotransmitters, Anal. Chim. Acta. 653 (2009) 1–22. doi:10.1016/j.aca.2009.08.038.

[23] R.J. Dinis-oliveira, Metabolomics of drugs of abuse : a more realistic view of the toxicological complexity, Bioanalysis. 6 (2014) 3155–3159. doi:10.4155/bio.14.260.

[24] E. Papaseit, M. Farré, F. Schifano, M. Torrens, Emerging drugs in Europe., Curr. Opin. Psychiatry. 27 (2014) 243–50. doi:10.1097/YCO.0000000000000071.

[25] M.H. Baumann, R.A. Glennon, J.L. Wiley, Neuropharmacology of New Psychoactive Substances (NPS), Current Topics in Behavioral Neurosciences, 2017.

[26] J.M. Prosser, L.S. Nelson, The Toxicology of Bath Salts: A Review of Synthetic Cathinones, J. Med. Toxicol. 8 (2012) 33–42. doi:10.1007/s13181-011-0193-z.

[27] M.H. Baumann, J.S. Partilla, K.R. Lehner, E.B. Thorndike, A.F. Hoffman, M. Holy, et al., Powerful Cocaine-Like Action of 3,4-Methylenedioxypyrovalerone (MDPV), a Principal Constituent of Psychoactive “Bath Salts” Products, Neuropsychopharmacology. 4 (2012) 552–62. doi:10.1038/npp.2012.204.

[28] R.A. Glennon, R. Young, Neurobiology of 3,4-methylenedioxypyrovalerone (MDPV) and α-pyrrolidinovalerophenone (α-PVP), Brain Res. Bull. 126 (2016) 111–126. doi:10.1016/j.brainresbull.2016.04.011.

[29] G.F. Koob, Neurobiology of Addiction Toward the Development of New Therapies, Ann. New York Acad. Sci. 909 (2000) 170–185.

[30] B. Yue, E. Pattison, W.L. Roberts, A.L. Rockwood, O. Danne, C. Lueders, et al., Choline in Whole Blood and Plasma: Sample Preparation and Stability, Clin. Chem. 54 (2008) 590–593.

[31] M. Coppola, R. Mondola, 3,4-Methylenedioxypyrovalerone (MDPV): Chemestry, pharmacology and toxicology of a new designer drug of abuse marketed online, Toxicol. Lett. 208 (2012) 12–15. doi:10.1016/j.toxlet.2011.10.002.

[32] K. Sivagnanam, D. Chaudari, P. López, M.E. Sutherland, V.K. Ramu, “Bath salts” induced severe reversible cardiomyopathy, Am. J. Case Rep. 14 (2013) 288–291. doi:10.12659/AJCR.889381.

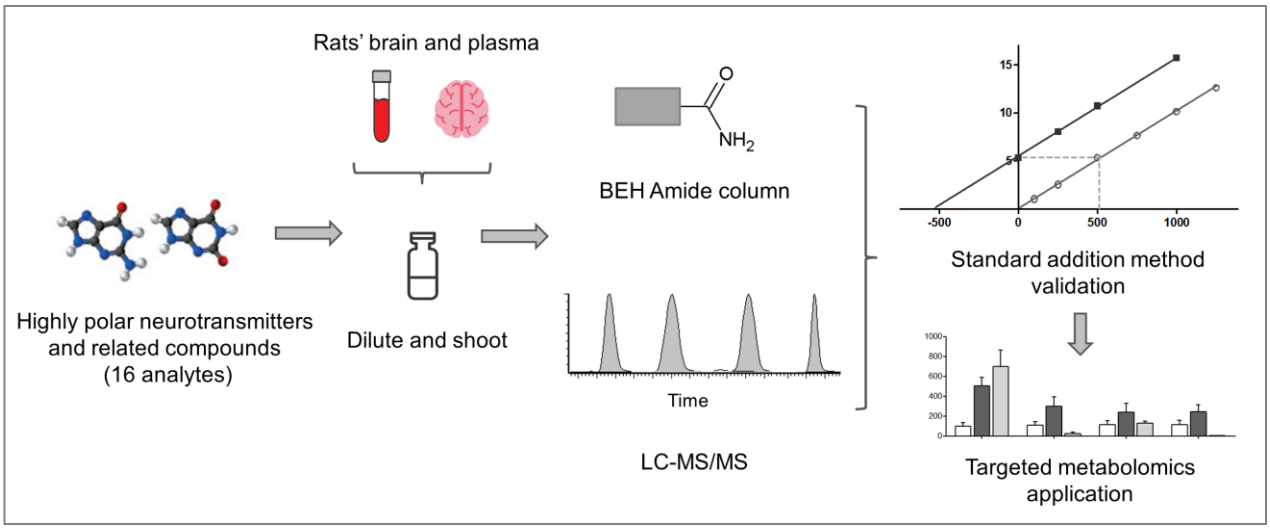
[33] F.L. Counselman, E.W. Mclaughlin, E.M. Kardon, A.S. Bhambhani-bhavnani, Creatine Phosphokinase Elevation in Patients Presenting to the Emergency Department With Cocaine-Related Complaints, Am. J. Emerg. Med. 071 (1997) 221–223.

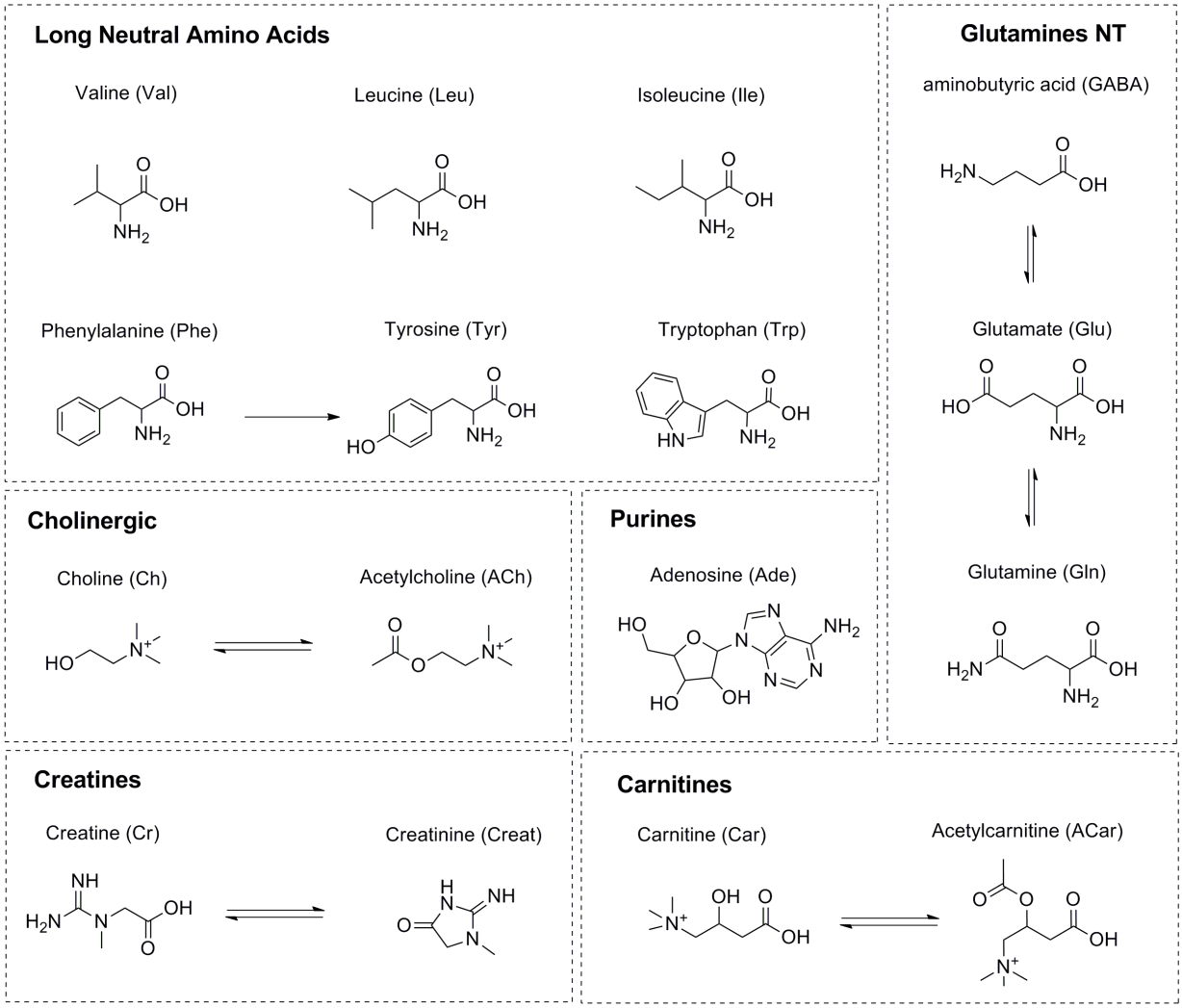
[34] R.D. Welch, K. Tood, G.S. Krause, Incidence of Cocaine-Associated Rhabdomyolysis, Ann. Emerg. Med. 155 (1991) 65–69.

[35] J.M. Wilson, M.E. Carroll, S.T. Lac, L.M. DiStefano, S.J. Kish, Choline acetyltransferase activity is reduced in rat nucelus accumbens after unlimited access to self-administration of cocaine, Neurosci. Lett. 180 (1994) 29–32.

[36] J. Chen, W. Hou, B. Han, G. Liu, J. Gong, Y. Li, et al., Target-based metabolomics for the quantitative measurement of 37 pathway metabolites in rat brain and serum using hydrophilic interaction ultra-high-performance liquid chromatography – tandem mass spectrometry, Anal Bioanal Chem. 408 (2016) 2527–2542. doi:10.1007/s00216-016-9352-z.

Graphical abstract





703

704 **Figure 1**. Chemical structures of the targeted analytes. Abbreviations: NT: neurotransmitters.

705

706

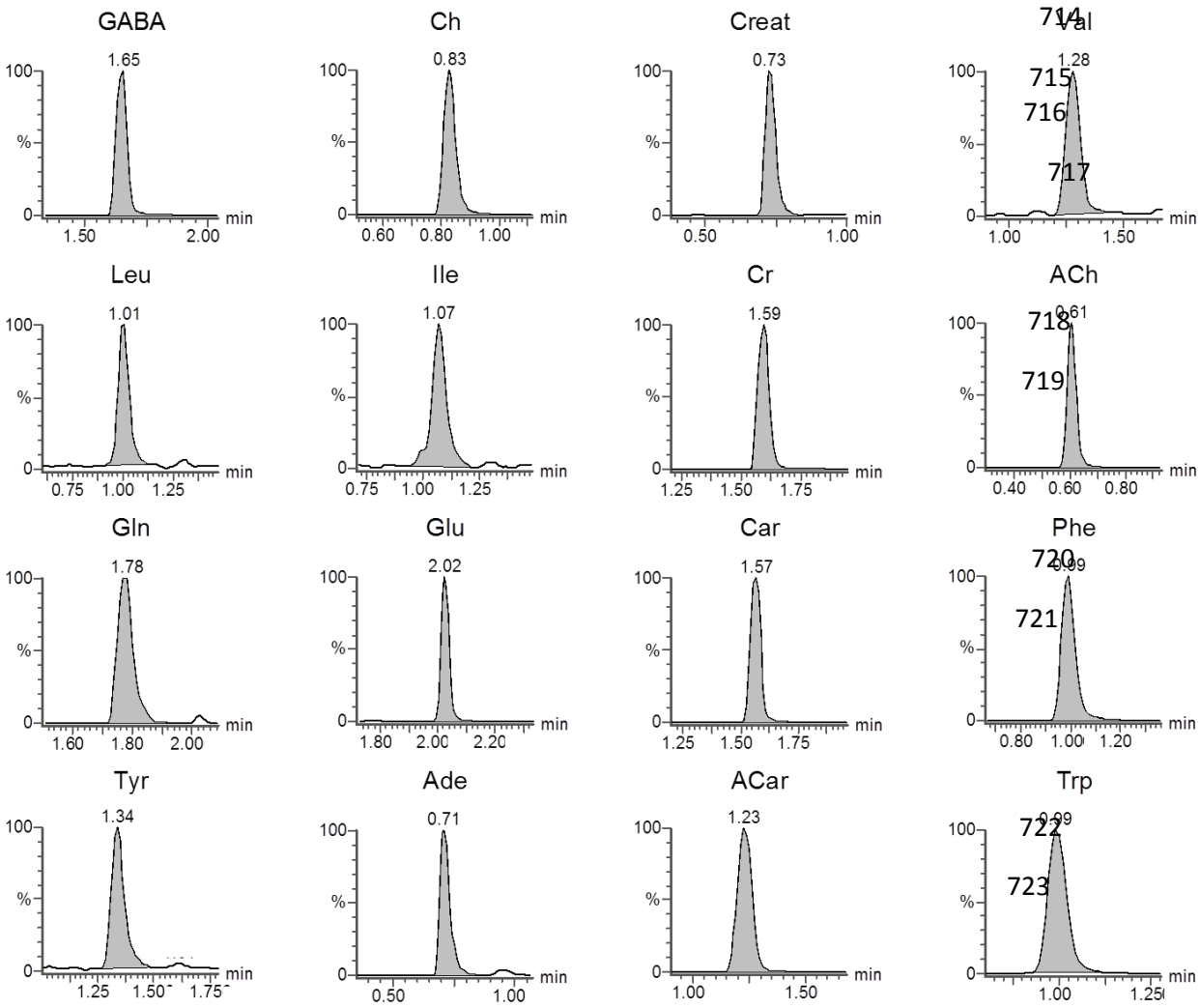
707

Compound	MW (g /mol)	Rt (min)	Precursor m/z	Species	Cone voltage (V)	Collision energy (eV)	Product m/z
GABA	103	1.65	104*	$[M+H]^+$	15	10	87*
							45
Ch	104	0.83	104*	$[M]^+$	15	10	45
							60*
Creat	113	0.73	114*	$[M+H]^+$	15	10	44*
							86
Val	117	1.28	118*	$[M+H]^+$	15	10	55
							72*
Leu	131	1.01	132*	$[M+H]^+$	15	10	86
						20	43*
Ile	131	1.07	132*	$[M+H]^+$	15	20	44
							69*
Cr	131	1.59	132*	$[M+H]^+$	15	10	90*
						20	44
ACh	146	0.61	146*	$[M]^+$	15	20	43
						10	87*
Gln	146	1.78	147*	$[M+H]^+$	15	10	130*
						20	84
Glu	147	2.02	148*	$[M+H]^+$	15	20	56
							84*
Car	161	1.57	162*	$[M]^+$	15	10	60
						20	43*
Phe	165	0.99	166*	$[M+H]^+$	15	10	120
						30	103*
Tyr	181	1.34	182*	$[M+H]^+$	15	20	136
						30	91*
Ade	267	0.71	268*	$[M+H]^+$	15	20	136*
						30	119
ACar	203	1.23	204*	$[M]^+$	15	10	60
						20	85*
Trp	204	0.99	205*	$[M+H]^+$	15	10	188*
						20	146
GABA-d_6	109	1.65	110*	$[M+H]^+$	15	10	93*
						20	49
Ch-d_4	108	0.83	108*	$[M]^+$	15	10	49
							60*
Creat-d_3	116	0.73	117*	$[M+H]^+$	15	10	47*
							89
^{13}C-^{15}N-Gln	148	1.78	149*	$[M+H]^+$	15	10	131*
						20	85
Glu-d_5	152	2.02	153*	$[M+H]^+$	15	10	60
						20	88*
Phe-d_5	170	0.99	171*	$[M+H]^+$	15	10	125
						30	106*
Tyr-d_4	185	1.34	186*	$[M+H]^+$	15	20	140
						30	94*
Trp-d_5	209	0.99	210*	$[M+H]^+$	15	10	192*
						20	150
$^{13}C_5$-Ade	272	0.71	273*	$[M+H]^+$	15	20	136*
						30	119
ACh-d_9	155	0.60	155*	$[M]^+$	15	10	87*
						20	43

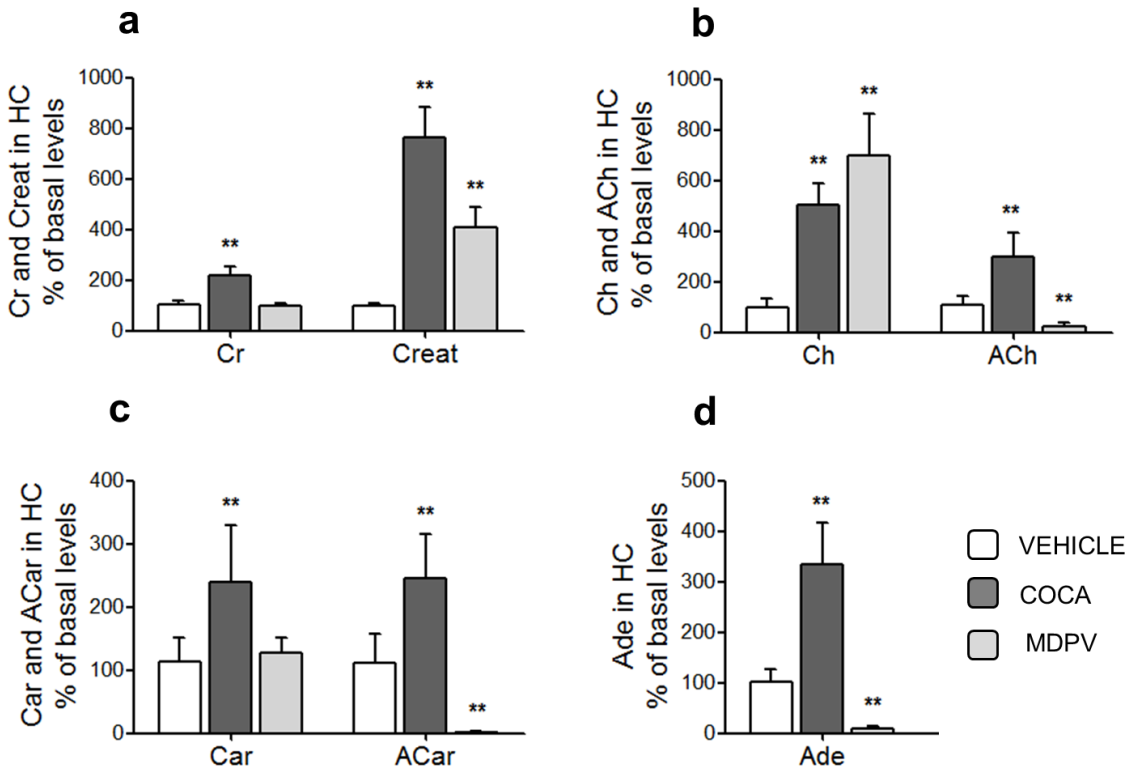
Table 1. Molecular weight (MW), retention times (Rt) and selected reaction monitoring (SRM) conditions for the detection of targeted analytes in rat brain and plasma. Transitions used for quantification are marked with an asterisk (*). Abbreviations: GABA, γ-aminobutyric acid; Ch, choline; Creat, creatinine; Val, valine; Leu, leucine; Ile, isoleucine; Cr, creatine; Ach, acetylcholine, Gln, glutamine; Glu, glutamate; Car, carnitine; Phe, phenylalanine; Tyr, tyrosine; Ade, adenosine; Acar, acetylcarnitine; Trp, tryptophan.

Compound	Internal standard	Range (ng/mL)	LOD (ng /mL)	Matrix	r^2	Intra-assay accuracy (%)	Inter-assay accuracy (%)	Intra-assay error (%RSD)	Inter-assay error (%RSD)	Matrix effect (%)	Matrix effect RSD (%)
GABA	GABA-d_6	25-5,000	5	Plasma	0.989	n.d.					
				Brain	0.988	105	95	15	19	10	20
Ch	Ch-d_4	25-20,000	4	Plasma	0.999	104	105	14	12	-9	7
				Brain	0.998	102	102	6	8	35	38
Creat	Creat-d_3	25-20,000	8	Plasma	0.999	105	100	7	8	0	28
				Brain	0.998	105	103	3	10	29	14
Val	$^{13}C_5$-Ade	50-20,000	50	Plasma	0.992	85	84	7	9	9	12
				Brain	0.994	98	103	8	12	5	15
Leu	Phe-d_5	25-5,000	25	Plasma	0.987	88	99	11	15	-6	17
				Brain	0.996	100	96	14	12	-4	12
Ile	Phe-d_5	25-20,000	10	Plasma	0.993	85	87	12	12	40	9
				Brain	0.996	90	89	4	16	25	14
Cr	Creat-d_3	25-10,000	20	Plasma	0.988	97	101	10	9	8	10
				Brain	0.987	105	101	17	15	-4	31
ACh	ACh-d_9	5-2,500	5	Plasma	0.993	n.d.					
				Brain	0.995	101	93	8	18	-18	37
Gln	^{13}C-^{15}N-Gln	25-20,000	6	Plasma	0.999	99	102	8	9	-21	11
				Brain	0.995	110	101	11	14	4	19
Glu	Glu-d_5	250-20,000	150	Plasma	0.998	98	105	10	16	17	16
				Brain	0.990	91	88	14	14	0	30
Car	Creat-d_3	25-20,000	5	Plasma	0.988	87	90	3	11	1	14
				Brain	0.989	103	107	9	19	35	25
Phe	Phe-d_5	50-20,000	5	Plasma	0.997	107	107	13	15	-9	11
				Brain	0.995	107	102	8	11	-3	17
Tyr	Tyr-d_4	100-20,000	5	Plasma	0.988	89	85	13	16	45	17
				Brain	0.990	95	93	15	24	20	9
Ade	$^{13}C_5$-Ade	25-20,000	5	Plasma	0.994	n.d.					
				Brain	0.994	111	105	6	12	4	8
ACar	Creat-d_3	25-5,000	5	Plasma	0.988	89	84	11	14	-19	17
				Brain	0.995	96	94	12	12	34	12
Trp	Trp-d_5	25-20,000	4	Plasma	0.997	107	112	13	15	-4	15
				Brain	0.991	95	92	7	20	-1	10

Table 2. Linearity, accuracy, precision, limit of detection (LOD) and matrix effect for the 16 analytes in plasma and brain homogenates.



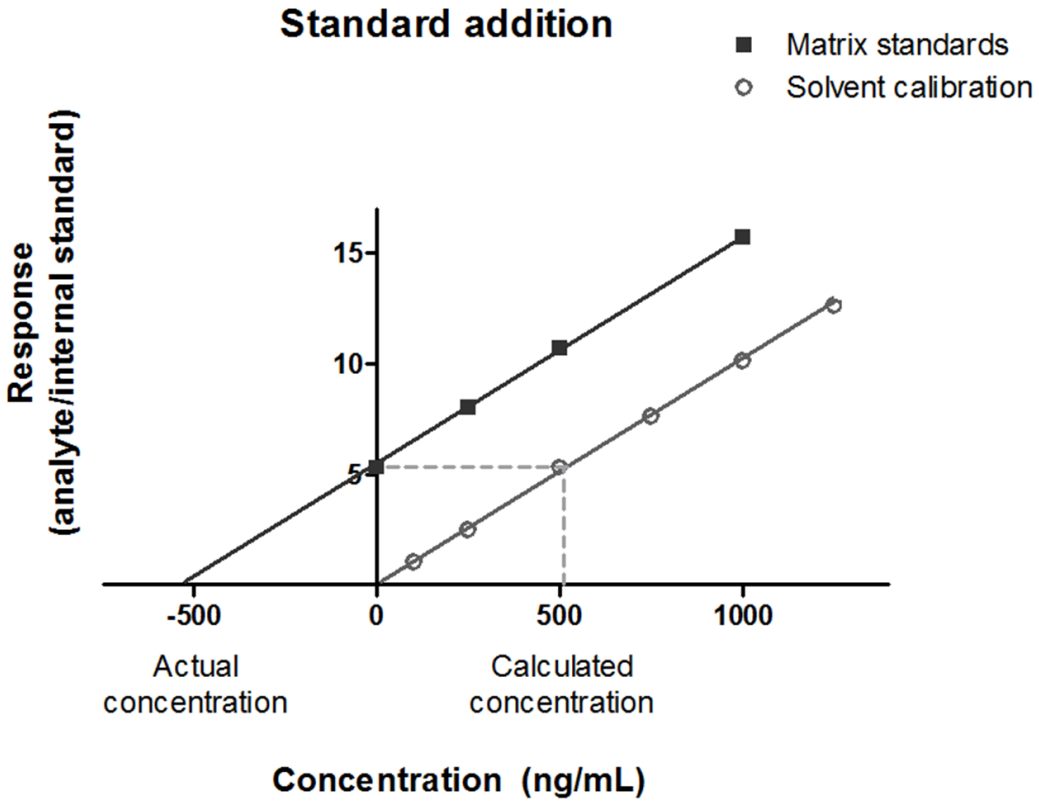
724 **Figure 2**. Chromatograms of the 16 targeted analytes in a real sample of rat hippocampus,
725 euthanized 1h after the intraperitoneal administration of cocaine (20 mg/kg). Brain
726 concentrations were: 14320 ng/mL for GABA, 3935 ng/mL for Ch, 865 ng/mL for Creat, 797
727 ng/mL for Val, 1127 ng/mL for Leu, 497 ng/mL for Ile, 1069 ng/mL for Cr (106900 ng/mL real
728 concentration), 35 ng/mL for ACh, 353 ng/mL for Gln (35300 ng/mL real concentration), 980
729 ng/mL for Glu (98000 ng/mL real concentration), 625 ng/mL for Car, 1289 ng/mL for Phe, 1778
730 ng/mL for Tyr, 17835 ng/mL for Ade, 141 ng/mL for ACar and 436 ng/mL for Trp. Abbreviations:
731 (γ-aminobutyric acid, GABA; choline, Ch; creatinine, Creat; valine, Val; leucine, Leu; isoleucine,
732 Ile; creatine, Cr; acetylcholine, Ach; glutamine, Gln; glutamate, Glu; carnitine, Car;
733 phenylalanine, Phe; tyrosine, Tyr; adenosine, Ade; acetylcarnitine, ACar; tryptophan, Trp).



734 **Figure 3.** Bars graph of the variations of the targeted analytes after the administration of
735 vehicle, cocaine (COCA) and methylenedioxypyrovalerone (MDPV) to male Wistar rats. a)
736 Alterations in the hippocampal concentrations of creatines (Cr and Creat), b) cholinergics (Ch
737 and ACh), c) carnitines (Car, ACar) and d) purines (Ade) were observed in comparison with the
738 vehicle administration. Abbreviations (creatine, Cr; creatinine, Creat; choline, Ch; acetylcholine,
739 Ach; carnitine, Car; acetylcarnitine, ACar; adenosine, Ade; and HC, hippocampus).

740

741



742

743 **Supplementary Figure 1.** Graphical calculation of the “actual” and “calculated” concentrations
744 using the standard addition method.

745

Compound	Matrix	Cycle 1 (%)	Cycle 2 (%)	Cycle 3 (%)
GABA	Brain	93±12	96±3	97±7
	Plasma	Not detected		
Ch	Brain	99±3	98±3	99±6
	Plasma	100±20	98±25	101±26
Creat	Brain	101±5	99±3	103±7
	Plasma	100±5	99±6	98±4
Val	Brain	107±15	98±8	106±12
	Plasma	104±7	103±8	99±3
Leu	Brain	106±13	99±8	108±10
	Plasma	105±9	104±9	101±6
Ile	Brain	105±15	99±8	106±10
	Plasma	104±10	105±6	104±5
Cr	Brain	104±10	101±4	109±8
	Plasma	100±6	99±7	96±3
ACh	Brain	98±4	98±3	100±6
	Plasma	Not detected		
Gln	Brain	99±4	99±2	100±5
	Plasma	102±5	100±6	100±5
Glu	Brain	101±11	96±4	99±9
	Plasma	105±10	100±8	100±7
Car	Brain	106±16	100±6	110±10
	Plasma	101±6	103±10	102±8
Phe	Brain	101±6	99±7	100±9
	Plasma	101±5	101±6	100±4
Tyr	Brain	94±16	89±27	87±20
	Plasma	108±23	110±18	106±19
Ade	Brain	95±8	97±3	99±6
	Plasma	Not detected		
ACar	Brain	99±6	97±4	100±8
	Plasma	99±10	100±7	101±5
Trp	Brain	100±5	96±6	95±6
	Plasma	99±7	97±8	99±8

Supplementary Table 1. Variations of the targeted analytes' concentrations in both brain and plasma with 3 freeze-thaw cycles (mean±SD values (expressed in %)) regarding the effect of the analytes' storage time, that it was evaluated up to 3 days at room temperature, 7 days at 4ºC and 14 days at -20ºC. The percentages correspond to the concentration obtained in cycle 1, 2, and 3, divided by concentration in cycle 0 and multiplied by 100.

COCAINE		Val	Leu	Ile	GABA	Glu	Gln	Phe	Tyr	Trp	Ch	ACh	Car	ACar	Cr	Creat	Ade
Brain	PFC - 1h	1.1	1.1	0.9	0.9	1.3	1.2	1.3	1.1	1.4	1.6	**1.5***	1.4	0.5	1.2	4.9	1.2
	Cer – 1h	1.2	1.2	1.1	1.2	**1.3****	1.2	1.5	1.2	1.2	**2.9****	**1.2****	**1.4***	0.5	1.3	**2.8****	0.8
	HC – 1h	**2.4****	**2.3****	**2.5****	**2.2****	**2.0****	2.0	2.4	2.7	2.7	**5.0****	**3.0****	**2.4****	**2.4***	**2.2****	**7.6****	**3.3****
	St - 1h	1.2	1.2	1.2	1.0	1.1	0.9	2.1	1.1	1.4	**3.0****	1.3	1.2	0.8	1.0	4.4	**2.1****
Peripheral	Plasma - 1h	2.2	1.4	1.2	n.d.	1.3	1.1	1.2	0.7	0.8	**1.5***	n.d.	0.9	0.5	**2.0***	**0.8***	n.d.

Supplementary table 2. Mean ratios (n=6) of all metabolites quantified in brain areas and plasma after the administration of COCA versus vehicle administration. Significant differences are underlined in bold as *P<0.05 and **P<0.01 (U-Mann Whitney test).

MDPV		Val	Leu	Ile	GABA	Glu	Gln	Phe	Tyr	Trp	Ch	ACh	Car	ACar	Cr	Creat	Ade
Brain	PFC - 1h	0.9	0.9	0.9	1.6	0.7	0.8	0.9	0.8	0.7	**4.8****	**0.3***	1.3	**0.0****	0.8	**6.4****	**0.1****
	Cer – 1h	1.1	1.0	1.1	**2.5****	0.9	0.9	1.2	1.2	1.1	**13.9****	**0.3****	**1.5***	**0.0****	1.1	**2.8****	**0.0****
	HC – 1h	0.9	0.9	0.9	**2.0****	**0.7***	0.8	0.9	1.0	0.8	**8.2****	**0.2****	1.3	**0.0****	1.0	**4.3****	**0.1****
	St - 1h	0.8	0.8	0.9	1.8	0.7	0.8	0.8	0.8	0.7	**5.5****	**0.3***	1.0	**0.0****	0.9	**3.9****	**0.1****
Peripheral	Plasma - 1h	0.5	1.1	1.1	n.d.	1.7	1.8	1.0	1.1	0.9	1.1	n.d.	1.2	0.9	1.2	**0.9***	n.d.

Supplementary table 3. Mean ratios (n=6) of all metabolites quantified in brain areas and plasma after the administration of MDPV versus vehicle administration. Significant differences are underlined in bold as *P<0.05 and **P<0.01 (U-Mann Whitney test).