Highly automated platform for simultaneous identification of monoamine-, amino acid- and peptide neurotransmitters by Hydrophilic Interaction Liquid Chromatography-Mass Spectrometry

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Neurons communicate via chemical signals called neurotransmitters (NTs). The numerous identified NTs can have very different physiochemical properties (solubility, charge, size etc.), so quantification of the various NT classes traditionally requires several analytical platforms/methodologies. We here report that a diverse range of NTs (e.g. peptides oxytocin and vasopressin, monoamines adrenaline and serotonin, and amino acid GABA) can be simultaneously identified/measured in small samples, using an analytical platform based on Hydrophilic Interaction Liquid Chromatography (HILIC) and high-resolution mass spectrometry (MS).

The highly automated platform is cost-efficient as manual sample preparation steps and one-

time-use equipment are kept to a minimum. Zwitter-ionic HILIC stationary phases were used for both on-line solid phase extraction (SPE) and capillary liquid chromatography (cLC); enabling compounds from all NT classes to elute in small volumes producing sharp and symmetric signals, and allowing precise quantifications of small samples (demonstrated with whole blood, 100 microliters per sample). An additional robustness-enhancing feature is automatic filtration/filter back-flushing (AFFL), allowing hundreds of samples to be analyzed without any parts needing replacement (due to e.g. clogged SPE or LC columns).
The platform can be installed by simple modification of a conventional LC-MS system, requiring one additional LC pump and one additional switching valve.

Introduction

NTs are metabolites used for communication between neurons in the brain. In humans, analysis of cerebrospinal fluid (CSF) gives the most direct measure of central NT levels. Due to their less invasive nature, neuroimaging approaches such as positron emission tomography (PET) and magnetic resonance (MR) spectroscopy [1,2] are promising alternatives. However, the cost, availability and practicality aspects of CSF extraction and neuroimaging have led many studies to rely on quantification of NTs from blood, saliva or other peripheral fluids.

Measuring levels of various NTs (e.g. GABA, serotonin and its precursor tryptophan, adrenaline, oxytocin and vasopressin) in blood or other bio-fluids gives valuable information for several purposes. Some studies point to differences in peripheral NT levels as potential biomarkers for psychiatric conditions (e.g. serotonin[3]), or use blood measures of NTs to validate experimental neuroscience methods[4,5]. Peripheral NT levels have also repeatedly been shown to correlate with e.g. parent-infant attachment (e.g. neuropeptides oxytocin and vasopressin[6]). Central oxytocin (OT) levels are thought to mediate the link between OT levels

in plasma and behaviour, although the highly limited ability of many NTs to cross the blood brain barrier render the relationship between central and peripheral levels less clear[7]. Therefore, reliable, valid and sensitive analytical methodologies are required to further establish the roles and predictive traits of peripheral NTs.

A further complication of employing peripheral NT levels is that reliability and validity can be questionable. Different methods of quantification do not always give comparable results. For OT, McCullough *et al*[8] recently pointed to a discrepancy of up to 100-fold between conventional radioimmunoassay (RIA) and the newer, commercially available enzyme immunoassay (EIA) approach. The authors suggest that such EIA measures may misattribute other molecules to OT. Moreover, RIA and EIA methodologies are intended for specific compounds, and can be very time-consuming to establish, reducing method flexibility for e.g. identifying novel metabolite candidates for brain/blood level correlations[9].

Another obstacle is the difficulty with obtaining convincing data from small amounts of sample. Recently, Clark *et al*[10] analysed cerebrospinal fluid (CSF) samples from human infants and showed a correlation between neuropeptide levels and behaviour. The authors were however unable to address the correspondence between plasma and CSF peptide levels, since the amount of blood required for conventional analysis of plasma OT exceeded the amount that could safely be drawn from neonates.

An alternative to RIA and EIA is MS based methods. MS provides detailed information of detected compounds, including molar mass, charge, and (clues to) its molecular structure (when employing tandem MS). MS is commonly coupled with liquid chromatography (LC), which separates compounds in a sample from each other, prior to MS detection. This step enhances e.g. sensitivity, as fewer compounds enter the MS per time (reducing ion suppression[11]). LC-MS is common instrumentation in modern analytical laboratories, and is

associated with excellent validity and high accuracy/precision measurements, especially when internal standards are used (e.g. adding known amounts of an isotopically labelled version of each analyte (e.g. the NTs) to the sample, which are used to correct for variances that can occur during sample preparation/LC-MS recording).

However, reported LC-MS methods for NT determinations are often intended for rather large amounts of sample [12-14] and may therefore not be suitable for analysis of limited, precious samples (from e.g. infants, blood banks, hair samples, etc.). Also, they are typically not suited for measuring all classes of NTs at once (due to the significant variation of physiochemical properties of NTs, such as hydrophobicity, charge etc.). Simultaneous determination of members from all NT classes would be desirable as NTs can interact extensively, as is well documented across human and non-human models. LC-MS methods may however also require a number of manual preparation steps (e.g. enriching the compounds with solid phase extraction (SPE) columns prior to LC-MS), thereby increasing analysis cost.

Our goal was to develop an LC-MS platform that would be capable of analysing samples to quantify all NT classes. Moreover, we wanted the platform to have high sensitivity, allowing analysis of minute samples. In addition, we wanted the platform to be automated, but at the same time very robust. Our proposed solution is to employ hydrophilic interaction liquid chromatography (HILIC)-mass spectrometry (MS)[15], with on-line solid phase extraction (SPE)[16] and automated filtration/filter backflushing (AFFL)[17]. HILIC-SPE is compatible with protein precipitation using acetonitrile (ACN) with 0.1 % formic acid (FA), allowing direct analysis of the supernatant with no additional steps. We will here describe the versatility of the method and demonstrate its sensitivity, quantification, reliability and other traits.

Results

Neurotransmitters/precursors with a wide range of physiochemical properties (see structures and other data, **Fig. 1**) could be identified/measured in a single analysis, needing only 100 µL of whole blood (see LC-MS chromatograms, **Fig. 2**). A novel, automated AFFL-SPE-cLC-MS analytical platform was designed for this purpose (see system illustration, **Fig. 3** and Supplementary Fig. S1). Evaluated with NT monoamines and amino acids with readily available labelled internal standards; see calibration curves and validation tables**, Fig. 4**, the automated platform could be used for reliable trace determinations. Due to the platform's sensitivity and versatility, trace levels of the much larger neuropeptides oxytocin and vasopressin could also be easily detected (**Fig. 2g** and **n**). The platform provided good and similar chromatographic quality for monoamines, amino acids and peptides (**Fig. 2**), suggesting that AFFL-SPE-cLC-MS is a suitable platform for comprehensive NT determinations in small samples.

Method development

Initially, two commercial zwitter-ionic HILIC columns for cLC separations were examined, (a) ZIC-HILIC[18] and (b) ZIC-cHILIC[19]. Both have zwitter-ionic stationary phases, but while (a) has a sulphobetaine type functionality (quaternary ammonium part = positive charge, sulphonic acid part = negative charge), where the negative charge is the most accessible to interacting solutes, (b) has a phosphorylcholine type functionality (phosphate part = negative charge, quaternary ammonium part = positive charge), where the positive charge is the most accessible. Neither of the two 15 cm long columns provided a baseline separation of all the NT analytes. However, since the analytes have distinct retention times, exact masses and/or MS/MS transitions (**Table 1**) and the platform provides satisfactory quantitative performance (see below), baseline separation was not considered to be a necessity. The efficiency (plate number N, see Supplementary note Calculation of efficiency), and asymmetry factor (A_s, see Supplementary Fig. S2, for details) of the platform were somewhat better with (b) (see N

values in caption of **Fig. 2**), hence the ZIC-cHILIC column was employed. Best resolution (R_s) and N were achieved with a mobile phase consisting of 70 % ACN and 30 % 100 mM ammonium formate at pH 3 (see Supplementary note Mobile phases, for brief discussion on other mobile phases examined). In addition, 300 µM ascorbic acid (anti-oxidant) was added to the mobile phase to avoid online-oxidation effects[20,21] (see Supplementary note Online oxidation).

An AFFL set-up[22] was used for on-line sample filtration (**Fig. 3, part A**), using a stainless steel filter, and subsequent back-flushing of the filter. This approach eliminated the need for single-use filters (and manual handling of these), thereby allowing for hundreds of whole blood sample injections without hardware replacement (including filter, columns etc.). On-line SPE[16] (**Fig. 3, part B**) was employed to automatically enrich the NTs prior to cLC separation, eliminating also the need for single-use SPE cartridges (and manual handling of these). Best performance was obtained using a ZIC-HILIC SPE column, compared to porous graphitic carbon- and ion exchange SPEs (see Supplementary note SPE column materials, and discussion below), with a 100 % ACN loading solvent (and two minute SPE loading time). At least 100 µL of acetonitrilic sample volumes (resulting from the protein precipitation step, see **Methods section** below) could be loaded on to the SPE without an analyte breakthrough occurring (see **Supplementary Fig. S3**). The cLC mobile phase successfully eluted the NT analytes from the SPE to the cLC column, and the analytes were chromatographed/detected in less than 9 minutes. Including the two-minutes loading time and one-minute post-run SPE re-equilibration time, the platform analysis time cycle was 12 minutes per sample.

The only sample preparation was a one-step ACN (with 0.1 % FA) precipitation, and this could easily be carried out in a 96-well format to further increase the throughput of the method.

Method validation

Viewing **Fig. 4** in more detail, the within-day and between-day repeatabilities (n=6 and n=5, respectively) were satisfactory for quantification (≤ 20 % relative standard deviation (RSD), as according to Federal Drug Agency (FDA) validation guidelines [23]) from lowest concentrations (between 0.05 nM and 250 nM, depending on the analytes expected endogenous levels) and up to three orders of magnitude, with good linearity (r^2 = 0.990 to 0.999) in both spiked blood and aqueous standard samples using the Orbitrap MS. Of the analytes investigated, serotonin had the lowest RSDs (within-day: 2 – 5 %, between day: 3 – 7 %) while adrenaline had the highest RSDs (within-day: 5 – 13 %, between-day: 14 – 17 %). In blood, the recoveries of the (spiked) neurotransmitters varied from 33 % for dopamine up to 91 % for serotonin, which were corrected for using isotope labelled internal standards added prior to sample preparation, which was one-step protein precipitation with ACN and 0.1 % FA, see **Methods section** below). Oxytocin and vasopressin (endogenously present at low pg/mL levels[8]) were also easily detected at quantifiable levels (i.e. signal to noise (S/N) >20) with the same sample preparation. The concentration limit of detection (cLOD) varied from 0.2 nM for GABA and adrenaline, up to 30 nM for tryptophan (see **Supplementary note Calculation of cLOD**).

The analyte stability in whole blood was demonstrated by re-analysing a number of compounds in samples after 1 week storage at 4° C and -20° C, after 24 h, 48 h and 1 week at room temperature, and after two freeze/thaw cycles. The analyte peak areas from the re-analyses were compared to the initial peak areas from day 1. At 4° C, - 20° C, and after 2 freeze-thaw cycles, the stability was satisfactory for all the neurotransmitters (i.e. no decrease in peak areas) in the sample. Serotonin and GABA were stable at room temperature for 48 h, but not 1 week, while the dopamine peak area was somewhat reduced after only 24 h at room temperature. The other neurotransmitters were stable at room temperature for at least 1 week.

In aqueous standards (diluted with ACN/H_2O, 70/30), some degree of instability was observed at room temperature and 4° C over night, hence fresh working solutions were made daily, and the stock solutions were stored at -80° C.

Discussion

Neurotransmitters/precursors could be identified/measured in a single analysis, needing only 100 µL of whole blood, using a novel automated AFFL-SPE-cLC-MS analytical platform.

Several LC-MS methods[12-14,24-26] have been reported for polar NT determination, but disadvantages can be the need for extensive manual sample preparation and large sample amounts, etc. For the more hydrophobic neuropeptides, standard reversed phase (RP) stationary phases have traditionally been used[27,28]. Disadvantages of such approaches are that they are limited to compounds of some hydrophobicity, and can therefore not be applied for small polar NT without the use of ion-pairing reagents (typically not compatible with MS) or chemical derivatization of the analytes (significantly adding to time spent on sample preparation). To our knowledge, no previous reports have demonstrated the ability to simultaneously handle both very hydrophilic and hydrophobic NTs.

Hence, key advantages of the presented AFFL-SPE-cLC-MS platform include simultaneous handling of polar NTs as well as neuropeptides with enhanced sensitivity (enabling analysis of small samples), a high degree of automation and increased speed.

The sensitivity is much attributed to the high-end mass Q-Exactive™ Orbitrap spectrometer[29] employed, but also to the use of capillary columns; using cLC reduces radial dilution of the analytes during chromatography compared to conventional columns[16], and employs lowered flow rates; both these features are associated with elevated sensitivity in ESI-MS[16,30]. Conventional bore HILIC columns (e.g. 1-2.1 mm IDs) would also be able to handle monoamines, amino acids and peptides simultaneously, but one should expect a decreased

sensitivity compared to using cLC. Although a ZIC-cHILIC column was chosen for this platform, the more common ZIC-HILIC column should perform nearly as well, but with some differences in selectivity[31] (i.e. the ability to separate various compounds).

Simultaneous determination of both polar analytes as well as neuropeptides would be far more challenging with the more common reversed phase LC approach, which separates according to hydrophobicity (typically limiting applicably to compounds with intermediate/low polarity). In contrast, HILIC columns separate compounds based on a number of factors, e.g. partitioning between an immobilized water layer in the stationary phase and the mobile phase, normal phase/adsorption interactions, electrostatic interactions, hydrogen bonding but also reversed-phase (RP) interactions[18,32-34]. The use of HILIC may also enhance sensitivity compared to RP, as relatively high amounts of ACN are used in the HILIC mobile phase, which can increase the MS signal[35].

Conventional cLC injections are limited to 5-10 µL injections. Here, we used on-line SPE to perform much larger injection volumes (100 µL) to lower the detection limits without compromising chromatography. On-line SPE also greatly reduces manual steps in sample preparation, which can be a central source of error in analysis[36]. On the other hand, on-line SPE-LC can be prone to clogging when handling complex samples[22]. To avoid this, the AFFL feature was employed, ensuring robust performance. AFFL-SPE-cLC is straightforward to install[17,22] and is compatible with common commercial LC-MS instrumentation (only requiring one extra pump and one switching valve). Whole blood samples are notorious for clogging LC equipment. We largely attribute the absence of problems analysing whole blood samples to the AFFL feature, which captures particulate matter with a filter that is back-flushed after each injection.

Using a zwitter-ionic HILIC phase also for SPE was superior to using ion exchange (IEX) phases or porous graphitic carbon (PGC) phases; IEX-HILIC and PGC-HILIC were

associated with reduced LC performance due to poor retention, or the ZIC-cHILIC separation column being incompatible with aqueous/very salty SPE loading solvents. This does not appear to be a significant issue when employing larger bore SPE-LC systems[37], but becomes a factor with small-bore systems (such as cLC), where system void volumes can play a larger role in system performance[38]. The "HILIC-HILIC" combination for SPE-LC has also been successfully used in proteomics and glycoproteomics[38,39]. To the authors´ knowledge, this is the first report on "HILIC-HILIC" SPE-LC for studying endogenous NTs/metabolites.

To ensure full analyte retention on the HILIC SPE, the aqueous standards were diluted with ACN/H_2O (70/30). Based on this, ACN with 0.1 % FA (for other precipitants examined, see **Supplementary note**) was used to precipitate proteins from the whole blood samples, in a 1+7.5 ratio (blood+precipitant). This allowed the blood samples to be directly injected onto the AFFL-SPE system, without any evaporation/re-dissolving step, making sample preparation both time-efficient and easy, with a minimum of manual work. Since whole blood could be analysed without plasmafication, additional time and manual work were saved, and the blood sample could be analysed less than one hour after the blood was collected.

When performing targeted determination and quantifications, triple quadrupole (3Q) instruments are often preferred, as they are known for their excellent quantitative capabilities. Orbitrap instruments are however superior[29] in resolution, and more typically used for untargeted analysis (e.g. comprehensive proteomics). This study exemplifies that precise quantifications of small metabolites can be successful with an Orbitrap instrument. In fact, linear calibration curves were obtained even without correction with internal standards (**Supplementary Fig. S4**), which implies relatively trustworthy quantifications for untargeted metabolomics as well. Considering that miniaturized HILIC-Orbitrap-based LC-MS systems work well for both proteomics[38, 39] and NTs (and likely other metabolites) as described here, it

is worth considering that a single instrument set-up similar to that described here can be used for both bottom-up proteomics and metabolomics, allowing their simultaneous determination.

Few methods on quantitative determination of NTs in human whole blood have been published, and reference materials are (to the authors' knowledge) not available. However, the endogenous levels quantified by our method were comparable (but somewhat higher) to levels reported in plasma samples (**Supplementary Table S2**). The reason is likely the high uptake of several neurotransmitters in blood platelets[40], and indeed the levels of some of the analytes were lower in plasma, especially serotonin (measured with a non-validated method).

We have demonstrated that AFFL-SPE-cLC-MS can be used for quantifying NTs in small amounts (a few drops) of blood, with minimal sample preparation only including ACN precipitation. The platform can be used for single-run chromatography of a wide range of NTs (monoamines, amino acids and peptides). Next steps will be to continue adding analytes to the platform, towards more comprehensive "neurotransmitter-omics", and validating the quantification of e.g. oxytocin, whose labeled internal standard has become commercially available at the time of submission (Sigma Aldrich, St. Louis, MO, USA). Therapeutics affecting NT activity may also be monitored simultaneously, as HILIC is compatible with drug analysis[41].

Methods

Study subjects and sample preparation

All subjects gave written informed consent, and the blood collection was approved by the Regional Ethics Committee (2011/1337/REK S-OE D). All methods were carried out in accordance with the approved guidelines and regulations.

Whole blood samples from veins were collected in vials containing K2 EDTA (BD, East Rutherford, NJ, USA), immediately aliquoted and stored at -80º C until the time of analysis. Aliquots of 100 µL of thawed whole blood were mixed with 50 µL of internal standard mixture (**Supplementary Table S3**), 100 µL of ACN/H_2O (70/30), 750 µL of cold ACN with 0.1 % FA (see Supplementary note Precipitants, for other precipitants examined), in 1.5 mL polypropylene vials (Eppendorf, Hamburg, Germany). The vials were vigorously vortexed for 20 seconds before kept at 4° C for 20 minutes, to maximize the protein precipitation. Finally, the vials were centrifuged for 10 minutes at 5000 rpm (Centrifuge 5415 R, Eppendorf). Approximately 700 µL of the supernatant were collected, and 100 µL of this were injected directly onto the AFFL-SPE-LC-MS system. Since only 100 µL is injected, it would be possible to start out with 50 µL of whole blood instead of 100 µL, but this would (to some degree) increase the uncertainty of the method due to difficulties with pipetting out very small volumes of whole blood. To increase the throughput of the method, the sample preparation could easily be done in a 96-well format.

AFFL-SPE-LC-MS System

For all chemicals and equipment used, see Supplementary Chemicals and equipment.

An automated filtration/filter backflush (AFFL)-SPE-LC-MS system[22] was used in this study (see **Supplementary Fig. S1** for an illustration of the entire system and **Fig. 3** for details of the switching system). In position 1 (load) the auto sampler injects 100 µL of sample, and then the sample is pumped through a 1 µm stainless steel filter fitted in a union. The analytes will pass through the filter and be retained on the SPE column, while un-retained non-polar compounds and solvent will go directly to waste. The purpose of the filter is to prevent larger particles from the sample e.g. precipitates, to reach and clog the SPE column. In position 2 (inject) the 10 port valve is switched and the LC pump elutes the analytes off the SPE column

and onto the analytical column. Simultaneously, the SPE pump connected to the auto-sampler will back-flush the filter and wash off particles/precipitates. For pump 1 the loading mobile phase was 100 % ACN, the flow rate was 75 µL/min and the loading time 2 minutes. For pump 2 the LC mobile phase consisted of 70 % ACN and 30 % 100 mM ammonium formate (pH 3, adjusted with 1 % formic acid), and the flow rate was 4 µL/min. The column temperature was 30° C. After being separated on the analytical column the analytes will be transferred to the ESI where they are ionized before they enter the Orbitrap MS (See Supplementary Table S1 for all MS parameters).

Method validation

The method was validated with samples made by spiking 100 µL of pooled in-house whole blood with 100 µL of the analyte STD mix (**Supplementary Table S3**) at seven different concentration levels: XL, L, LM, M, HM, H and XH (**Supplementary Table S4**). 50 µL of the internal standard mix (**Supplementary Table S3**) were added to all of the samples before 750 µL of cold ACN with 0.1 % FA were rapidly added. The rest of the procedure was the same as for the sample preparation, see section above. The calibration samples were made in the same way as the validation samples, and all samples were analysed on the same day as they were prepared. The within-day repeatabilities were found by analysing 6 sample replicates of validation samples with L, M and H concentration levels on the same day, while validation samples with L, M and H concentrations were analysed on 5 different days to investigate the between-day repeatabilities. The linearity was examined with standard solutions (n = 3) and with validation samples (n = 7). The determination of the cLOD was limited by the lack of blood without NTs (blank matrix). However, a crude estimate was calculated by extrapolation (for details, see **Supplementary note Calculations of cLOD**).

The recovery was investigated in the following way: one pooled blood sample was spiked with NT analytes before protein precipitation, and another pooled blood sample was spiked with NT analytes after protein precipitation. To calculate the recovery, equation (1) was used. A is the peak area of the analyte and A_{is} is the peak area of the internal standard, respectively.

$$RECOVERY = 100\% \times \left(\frac{\frac{A}{A_{is}} post-spike}{\frac{A}{A_{is}} pre-spike} \right) \qquad (1)$$

Stability was tested at room temperature, 4 ° C, -20 °C and with thawing/freezing. Validation samples from the first validation day were re-analysed after 24 h, 48 h, 1 week and after one and two thaw/freeze cycles, and peak areas were compared with that of day 1.

Quantification of neurotransmitters in whole blood

To quantify the amounts of NTs in the whole blood samples, calibration curves based on calibration samples with concentrations from XL to XH (Supplementary Table S4), constructed using Excel, were used. Since the calibration solutions were made by spiking blood which already contained the neurotransmitters of interest, the endogenous levels of NTs were calculated using the regression equation from the calibration curve, corrected for the endogenous level (for details, see **Supplementary note Calculation of NT concentrations, and Supplementary Fig. S5**).

References

1 Henriksen, G. & Willoch, F. Imaging of opioid receptors in the central nervous system. *Brain* **131**, 1171-1196 (2008).

2 Stagg, C. J., Bachtiar, V. & Johansen-Berg, H. The role of GABA in human motor learning. *Curr. Biol.* **21**, 480-484 (2011).

3 Hughes, C. W., Petty, F., Sheikha, S. & Kramer, G. L. Whole-blood serotonin in children and adolescents with mood and behavior disorders. *Psychiatry Res.* **65**, 79-95 (1996).

4 Walderhaug, E. *et al.* Interactive effects of sex and 5-HTTLPR on mood and impulsivity during tryptophan depletion in healthy people. *Biol. Psychiatry* **62**, 593-599 (2007).

5 Bart, G. *et al.* Nalmefene Induced Elevation in Serum Prolactin in Normal Human Volunteers: Partial Kappa Opioid Agonist Activity? *Neuropsychopharmacology* **30**, 2254-2262 (2005).

6 Feldman, R. Oxytocin and social affiliation in humans. *Horm. Behav.* **61**, 380-391 (2012).

7 Neumann, I. D., Maloumby, R., Beiderbeck, D. I., Lukas, M. & Landgraf, R. Increased brain and plasma oxytocin after nasal and peripheral administration in rats and mice. *Psychoneuroendocrinology* **38**, 1985-1993 (2013).

8 McCullough, M. E., Churchland, P. S. & Mendez, A. J. Problems with measuring peripheral oxytocin: can the data on oxytocin and human behavior be trusted? *Neurosci. Biobehav. Rev.* **37**, 1485-1492 (2013).

9 Weisman, O. & Feldman, R. Oxytocin administration affects the production of multiple hormones. *Psychoneuroendocrinology* **38**, 626 (2013).

10 Clark, C. L. *et al.* Neonatal CSF oxytocin levels are associated with parent report of infant soothability and sociability. *Psychoneuroendocrinology* **38**, 1208-1212 (2013).

11 Annesley, T. M. Ion suppression in mass spectrometry. *Clin. Chem.* **49**, 1041-1044 (2003).

12 Gosetti, F., Mazzucco, E., Gennaro, M. C. & Marengo, E. Simultaneous determination of sixteen underivatized biogenic amines in human urine by HPLC-MS/MS. *Anal. Bioanal. Chem.* **405**, 907-916 (2013).

13 Wei, B. *et al.* Determination of monoamine and amino acid neurotransmitters and their metabolites in rat brain samples by UFLC–MS/MS for the study of the sedative-hypnotic effects observed during treatment with S. chinensis. *J. Pharm. Biomed. Anal.* **88**, 416-422 (2014).

14 Lee, J., Huang, B. X., Yuan, Z. & Kim, H.-Y. Simultaneous determination of salsolinol enantiomers and dopamine in human plasma and cerebrospinal fluid by chemical derivatization coupled to chiral liquid chromatography/electrospray ionization-tandem mass spectrometry. *Anal. Chem.* **79**, 9166-9173 (2007).

15 Nguyen, H. P. & Schug, K. A. The advantages of ESI-MS detection in conjunction with HILIC mode separations: Fundamentals and applications. *J. Sep. Sci.* **31**, 1465-1480 (2008).

16 Rogeberg, M., Malerod, H., Roberg-Larsen, H., Aass, C. & Wilson, S. R. On-line solid phase extraction–liquid chromatography, with emphasis on modern bioanalysis and miniaturized systems. *J. Pharm. Biomed. Anal.* **87**, 120-129 (2014).

17 Svendsen, K. O. *et al.* Automatic filtration and filter flush for robust online solid-phase extraction liquid chromatography. *J. Sep. Sci.* **34**, 3020-3022 (2011).

18 Hemström, P. & Irgum, K. Hydrophilic interaction chromatography. *J. Sep. Sci.* **29**, 1784-1821 (2006).

19 Jiang, W., Fischer, G., Girmay, Y. & Irgum, K. Zwitterionic stationary phase with covalently bonded phosphorylcholine type polymer grafts and its applicability to separation of peptides in the hydrophilic interaction liquid chromatography mode. *J. Chromatogr. A* **1127**, 82-91 (2006).

20 Kertesz, G. J. V. B. & Vilmos. Using the electrochemistry of the electrospray ion source. *Anal. Chem.* **79**, 5510-5520 (2007).
21 Plattner, S., Erb, R., Chervet, J.-P. & Oberacher, H. Ascorbic acid for homogenous redox buffering in electrospray ionization–mass spectrometry. *Anal. Bioanal. Chem.* **404**, 1571-1579 (2012).
22 Roberg-Larsen, H. *et al.* High sensitivity measurements of active oxysterols with automated filtration/filter backflush-solid phase extraction-liquid chromatography–mass spectrometry. *J. Chromatogr. A* **1255**, 291-297 (2012).
23 *Guidance for Industry: Bioanalytical Method Validation*,(2013) Available at: www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm368107.pdf (Accessed 18/06/2014).
24 Zhang, X. *et al.* Capillary hydrophilic interaction chromatography/mass spectrometry for simultaneous determination of multiple neurotransmitters in primate cerebral cortex. *Rapid Commun. Mass Spectrom.* **21**, 3621-3628 (2007).
25 Carrera, V., Sabater, E., Vilanova, E. & Sogorb, M. A. A simple and rapid HPLC–MS method for the simultaneous determination of epinephrine, norepinephrine, dopamine and 5-hydroxytryptamine: Application to the secretion of bovine chromaffin cell cultures. *J. Chromatogr. B* **847**, 88-94 (2007).
26 Tareke, E., Bowyer, J. F. & Doerge, D. R. Quantification of rat brain neurotransmitters and metabolites using liquid chromatography/electrospray tandem mass spectrometry and comparison with liquid chromatography/electrochemical detection. *Rapid Commun. Mass Spectrom.* **21**, 3898-3904 (2007).
27 Mabrouk, O. S. & Kennedy, R. T. Simultaneous oxytocin and arg-vasopressin measurements in microdialysates using capillary liquid chromatography–mass spectrometry. *J. Neurosci. Methods* **209**, 127-133 (2012).
28 Zhang, G., Zhang, Y., Fast, D. M., Lin, Z. & Steenwyk, R. Ultra sensitive quantitation of endogenous oxytocin in rat and human plasma using a two-dimensional liquid chromatography–tandem mass spectrometry assay. *Anal. Biochem.* **416**, 45-52 (2011).
29 Michalski, A. *et al.* Mass spectrometry-based proteomics using Q Exactive, a high-performance benchtop quadrupole Orbitrap mass spectrometer. *Mol. Cell. Proteomics* **10**, M111. 011015, doi:10.1074/mcp.M111.011015 (2011).
30 Cole, R. B. *Electrospray ionization mass spectrometry: fundamentals, instrumentation, and applications*. (Wiley, 1997).
31 Zhang, R. *et al.* Evaluation of mobile phase characteristics on three zwitterionic columns in hydrophilic interaction liquid chromatography mode for liquid chromatography-high resolution mass spectrometry based untargeted metabolite profiling of Leishmania parasites. *J. Chromatogr. A* **1362**, 168-179 (2014).
32 Alpert, A. J. Hydrophilic-interaction chromatography for the separation of peptides, nucleic acids and other polar compounds. *J. Chromatogr. A* **499**, 177-196 (1990).
33 Guo, Y. & Gaiki, S. Retention behavior of small polar compounds on polar stationary phases in hydrophilic interaction chromatography. *J. Chromatogr. A* **1074**, 71-80 (2005).
34 McCalley, D. V. Study of the selectivity, retention mechanisms and performance of alternative silica-based stationary phases for separation of ionised solutes in hydrophilic interaction chromatography. *J. Chromatogr. A* **1217**, 3408-3417 (2010).
35 Periat, A., Boccard, J., Veuthey, J.-L., Rudaz, S. & Guillarme, D. Systematic comparison of sensitivity between hydrophilic interaction liquid chromatography and reversed phase liquid chromatography coupled with mass spectrometry. *J. Chromatogr. A* **1312**, 49-57 (2013).
36 Majors, R. An overview of sample preparation. *LC GC* **9**, 16-20 (1991).
37 Røen, B. T., Sellevåg, S. R. & Lundanes, E. On-line solid phase extraction-liquid chromatography–mass spectrometry for trace determination of nerve agent degradation products in water samples. *Anal. Chim. Acta* **761**, 109-116 (2013).

38 Malerod, H., Rogeberg, M., Tanaka, N., Greibrokk, T. & Lundanes, E. Large volume injection of aqueous peptide samples on a monolithic silica based zwitterionic-hydrophilic interaction liquid chromatography system for characterization of posttranslational modifications. *J. Chromatogr. A* **1317**, 129-137 (2013).
39 Di Palma, S., Boersema, P. J., Heck, A. J. & Mohammed, S. Zwitterionic hydrophilic interaction liquid chromatography (ZIC-HILIC and ZIC-cHILIC) provide high resolution separation and increase sensitivity in proteome analysis. *Anal. Chem.* **83**, 3440-3447 (2011).
40 Collins, C. M., Kloek, J. & Elliott, J. M. Parallel changes in serotonin levels in brain and blood following acute administration of MDMA. *J. Psychopharmacol.* **27**, 109-112 (2013).
41 Hsieh, Y. Potential of HILIC-MS in quantitative bioanalysis of drugs and drug metabolites. *J. Sep. Sci.* **31**, 1481-1491 (2008).

Author contributions

E.F.J., S.R.W. and E.L. planned and designed the experiments. E.F.J. performed the experiments and data analysis. E.F.J., S.R.W. and S.L. wrote the manuscript. All authors reviewed and approved the paper.

Additional information

Competing financial interests: The authors declare no competing financial interests.

Figure legends

Figure 1: Neurotransmitter molecular structures and info. Molecular structures of the six small neurotransmitters and the amino acid sequence of the two neuropeptides. A table

with molar mass (M_m), octanol-water partition coefficient (log P) and pK_a values of all analytes are included (values obtained from SciFinder (CAS, Columbus, OH, USA)).

Figure 2: LC-MS chromatograms of neurotransmitters identified in whole blood sample. Chromatograms showing the NTs identified/measured in a whole blood sample from a healthy volunteer with no medical history of importance. The NT peak is compared with the internal standard (IS) peak and the retention times are included. a) GABA (plate number N = 16 000), b) GABA-IS, c) PEA (N = 12 000), d) PEA-IS, e) dopamine (no N calculated), f) dopamine-IS, g) oxytocin (N = 6000, no IS available), h) serotonin (N = 6000), i) serotonin-IS, j) adrenaline (N = 19 000), k) adrenaline-IS, l) tryptophan (N = 4000), m) tryptophan-IS and n) vasopressin (N = 10 000, no IS available).

Figure 3: Illustration of AFFL-SPE-cLC-MS system. The two switching positions of the AFFL-SPE system, (1) "load" and (2) "inject" are illustrated. The shadowed areas A and B highlight the AFFL filter and the SPE, respectively.

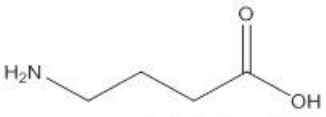
Figure 4: Validation data. Calibration curves for six NTs in spiked whole blood, including regression equations and R^2 values. The non-zero values for b in "y = ax + b" are due to endogenous levels of the neurotransmitters in blood. The table to the right show validation data including linear range, R^2 values (in blood and standards), concentration limits of detection (cLOD), minimum detectable amounts (MDs) and recovery (%). The table to the left shows the within-day and between-day repeatabilities of the NTs in spiked whole blood at the concentration levels L (low), M (medium) and H (high).

* No result for dopamine was obtained at the concentration level L, due to s/n < 10.

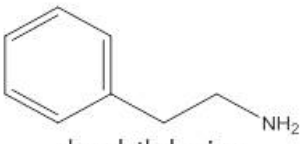
Table 1: MS/MS transitions and retention times. The table presents the MS/MS transitions for all the NTs examined in this study, and for the internal standards of the six small NTs. Isotope-labelled internal standards for the neuropeptides were not available at the time the study was conducted. Retention times are also included.

*No considerable fragmentation for vasopressin was observed with fragmentation energy of 25 or 35 %, so the precursor ion *m/z* (1084.455) was used for identification.

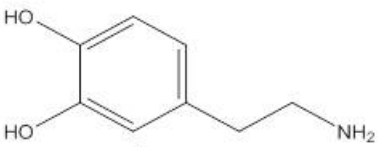
	Analyte		**Internal standard**		
Neurotransmitter	Precursor ion *(m/z)*	Product ion *(m/z)*	Precursor ion *(m/z)*	Product ion *(m/z)*	Retention time (min)
GABA	104.07	87.0443	106.09	89.0569	6.9
PEA	122.10	105.0699	127.13	110.1012	4.0
Dopamine	154.09	137.0593	158.11	141.0843	6.2
Serotonin	177.09	160.0751	181.13	164.1003	4.8
Adrenaline	184.10	166.0856	190.13	172.1233	6.7
Tryptophan	205.10	188.0699	208.12	191.0887	4.7
Oxytocin	1007.40	723.26	-	-	5.4
Vasopressin	1084.45	*	-	-	8.4



gamma-aminobutyric acid
(GABA)

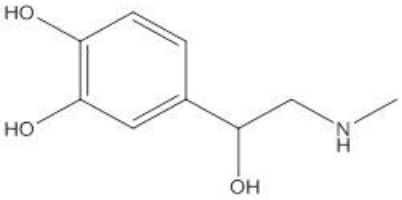


phenylethylamine
(PEA)

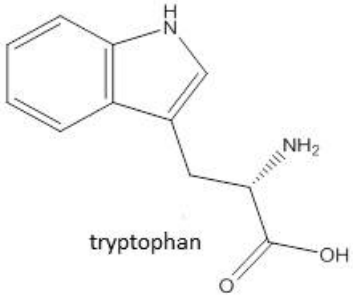


dopamine

serotonin



adrenaline

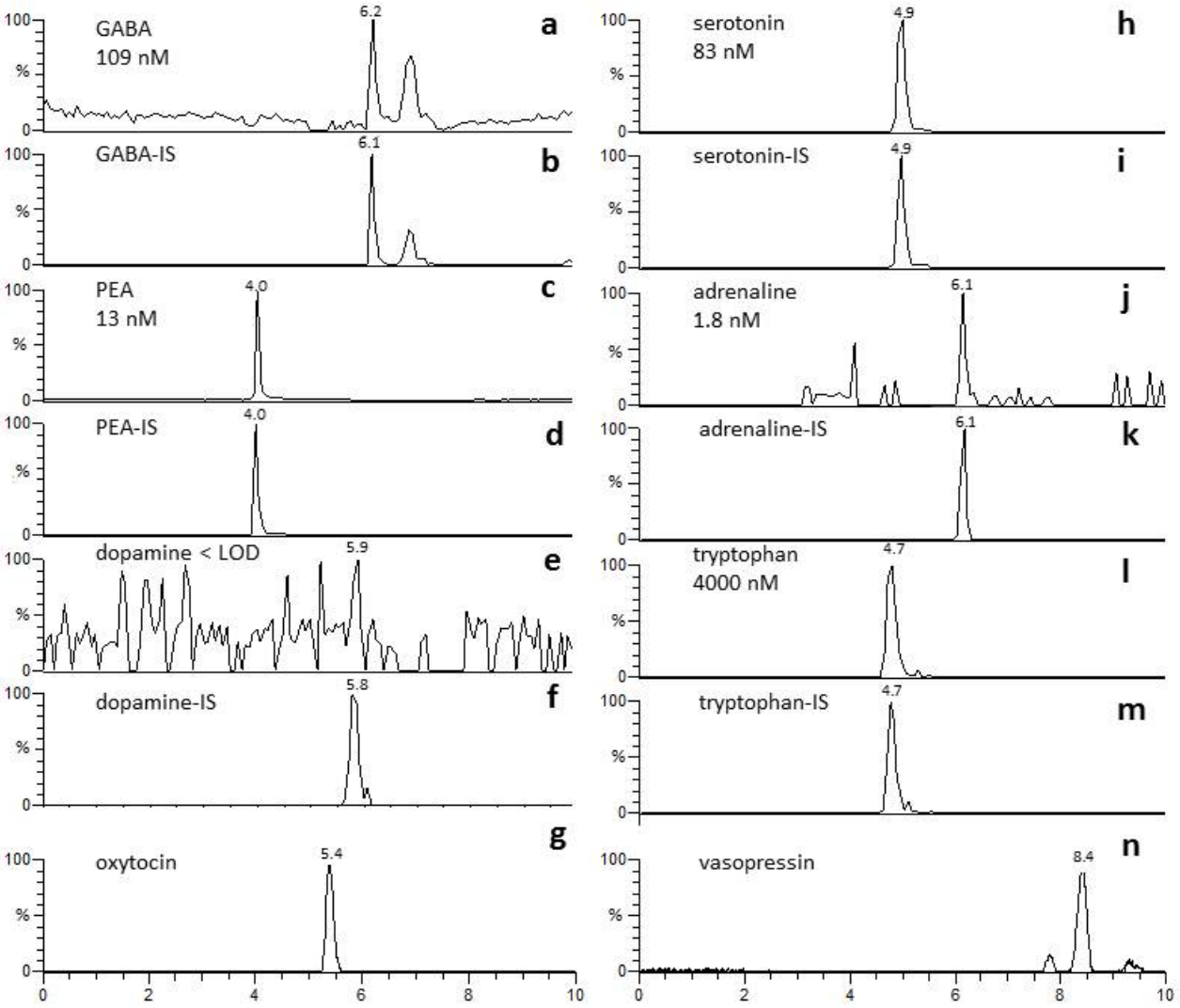


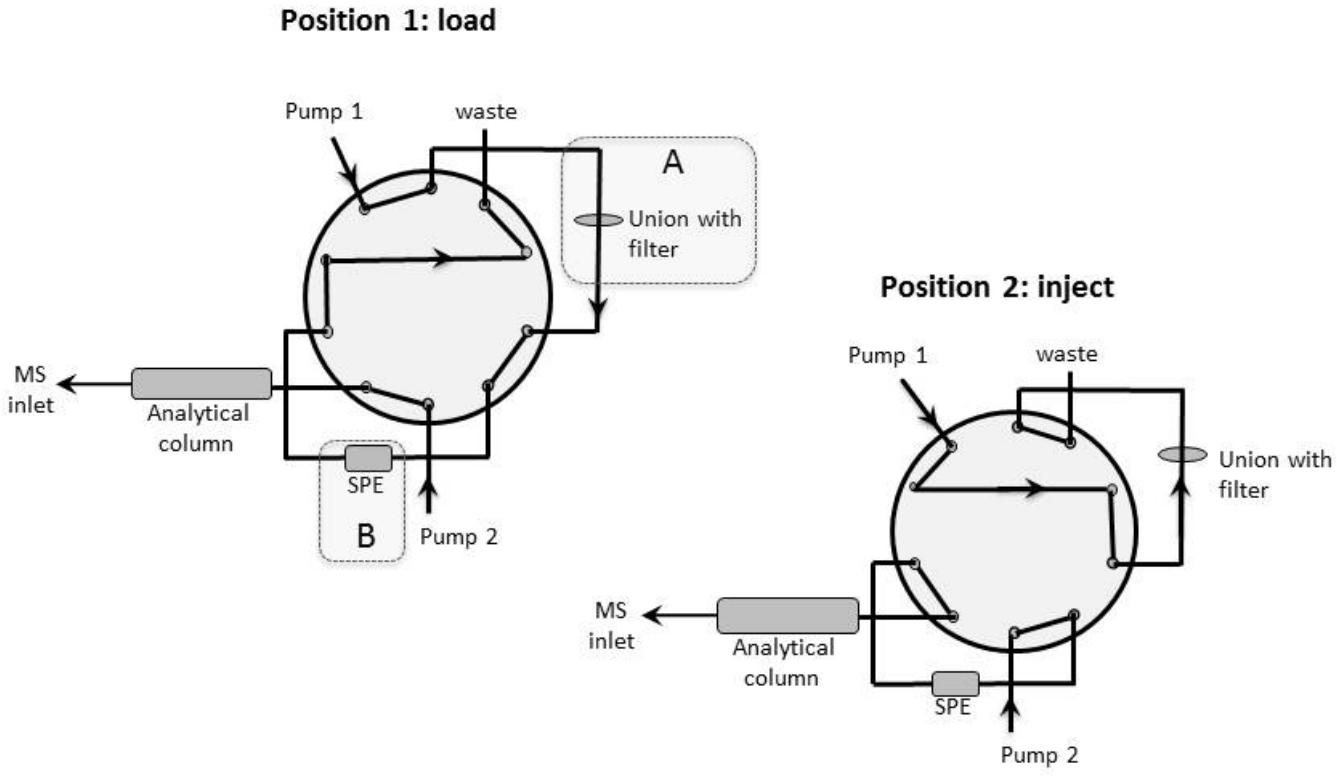
tryptophan

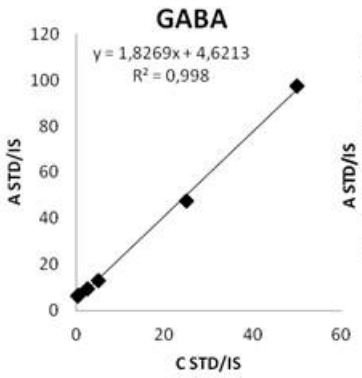
oxytocin: Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly

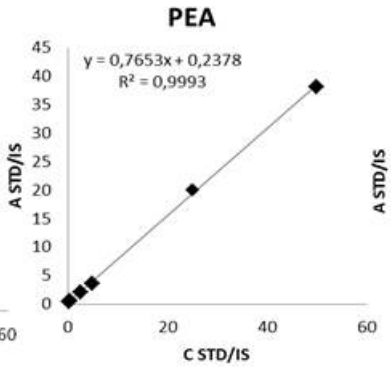
vasopressin: Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly

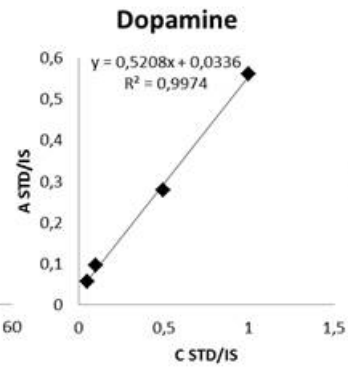
	GABA	PEA	Dopamine	Serotonin	Adrenaline	Tryptophan	Oxytocin	[Arg^8]-Vasopressin
M_m	103.12	121.18	153.18	176.22	183.20	204.23	1007.19	1084.23
LogP	- 0.64	1.44	0.046	0.55	-0.54	0.70	-1.23	-4.11
pK_a (base)	11.3	9.9	10.1	9.5	9.2	9.7	6.1	13.8
pK_a (acid)	4.4					2.3		

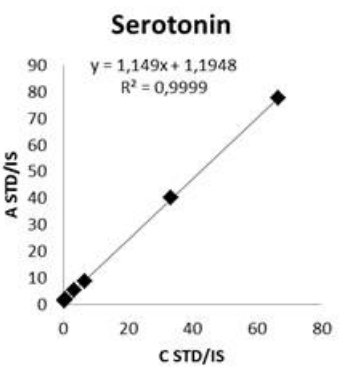

GABA
109 nM
6.2
a
GABA-IS
6.1
b
PEA
13 nM
4.0
c
PEA-IS
4.0
d
dopamine < LOD
5.9
e
dopamine-IS
5.8
f
oxytocin
5.4
g
serotonin
83 nM
4.9
h
serotonin-IS
4.9
i
adrenaline
1.8 nM
6.1
j
adrenaline-IS
6.1
k
tryptophan
4000 nM
4.7
l
tryptophan-IS
4.7
m
vasopressin
8.4
n
100
%
0
0
2
4
6
8
10

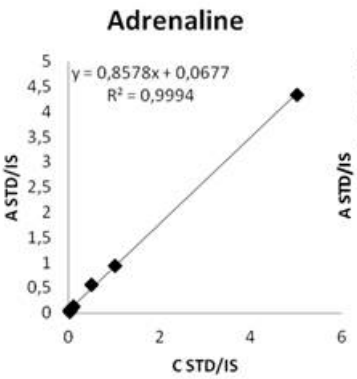

Position 1: load
Pump 1
waste
A
Union with filter
MS inlet
Analytical column
SPE
B
Pump 2
Position 2: inject
Pump 1
waste
Union with filter
MS inlet
Analytical column
SPE
Pump 2

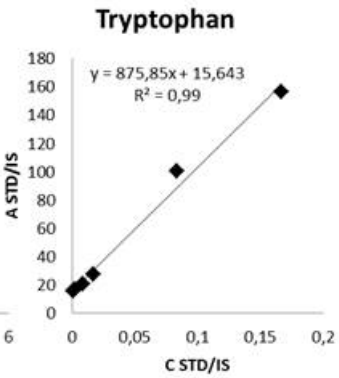












	Within-day repeatability (n = 6)			Between-day repeatability (n = 5)		
	Mean A/Ais	SD	RSD %	Mean A/Ais	SD	RSD %
GABA						
L	7.0	0.3	4	6.9	0.5	7
M	14.5	0.6	4	14	1	7
H	88	4	5	92.6	7.1	8
PEA						
L	0.6	0.5	8	0.53	0.06	12
M	3.2	0.2	5	3.8	0.4	11
H	31	1	4	35	3	9
Dopamine						
L*	-	-	-	-	-	-
M	0.10	0.09	9	0.09	0.08	9
H	0.60	0.04	8	0.556	0.009	2
Serotonin						
L	1.58	0.06	4	1.71	0.04	3
M	8.4	0.4	5	8.9	0.6	7
H	77	1	2	81	4	5
Adrenaline						
L	0.12	0.02	13	0.09	0.02	16
M	0.45	0.06	13	0.11	0.02	17
H	0.92	0.05	5	0.8	0.1	14
Tryptophan						
L	16	1	7	17	1	7
M	29	2	6	29	4	13
H	167	11	7	174	15	8

	Linear range nM	R^2 Blood (n = 6)	R^2 STD (n=3)	cLOD (nM)	MD (pmol)	Recovery %
GABA	5-5000	0.998	0.998	0.2	0.02	42
PEA	5-5000	0.999	0.999	1	0.1	62
Dopamine	0.05-50	0.997	0.998	1	0.1	33
Serotonin	5-5000	0.999	0.999	1	0.1	91
Adrenaline	0.05-50	0.992	0.99	0.2	0.02	57
Tryptophan	250-250000	0.99	0.998	30	3	52

Highly automated platform for simultaneous identification of monoamine-, amino acid- and peptide neurotransmitters by Hydrophilic Interaction Liquid Chromatography-Mass Spectrometry

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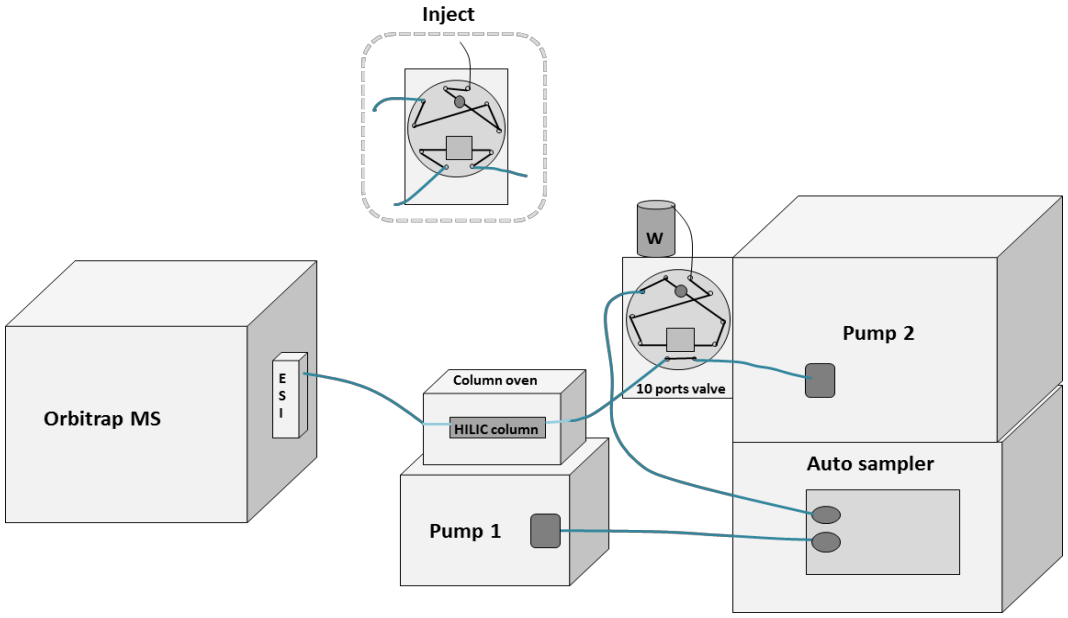
Chemicals and equipment

Mobile phases were made with HPLC grade acetonitrile (ACN) from VWR (West Chester, PA, USA), and HPLC water (Chromasolv Plus for HPLC), ammonium formate (NH_4HCO_2), formic acid (FA, 98 %), and ascorbic acid, all purchased from Sigma Aldrich (St. Louis, MO, USA). Tracy SPE columns (ZIC-HILIC, SCX, WCX, PGC, 0.5 (ID) x 5 mm) were obtained from G&T Septech (Kolbotn, Norway), while the ZIC-HILIC and the ZIC-cHILIC analytical columns (0.3 mm x 150 mm, 3.5 µm) were from Merck Millipore (Merck KGaA, Darmstadt, Germany). Two pumps were used: a Hitachi L-7110 (Merck) as SPE pump, and an Agilent 1100 series capillary gradient pump (Agilent Technologies, Palo Alto, CA, USA) with a max 20 µL/min flow cell, as LC pump. Injection was performed by a G1313 A ALS standard auto sampler (Agilent Technologies). A 10 port two-position switching valve (1/16", 0.25 mm bore) from Valco (Houston, TX, USA) controlled by the LC pump's Chemstation software (Agilent), performed the column switching. The AFFL filter was of stainless steel (1/16 µm, 1 µm screen) and fitted in a union (1/16 µm, 0.25 mm bore), both obtained from Valco. The column was connected to a Q-Exactive Orbitrap mass spectrometer (Thermo Scientific, Bellafonte, PA, USA) with a capillary electrospray ionization source (ESI, Thermo). The ESI was operated in positive ionization mode with a capillary voltage of 2500 V. Mass spectra were acquired in the *m/z* range 80-210. The transitions between the precursor ions to the most intensive fragment ions were monitored for qualitative determination of each NT in targeted MS/MS mode. XCalibur software was used for controlling the MS and for data collection.

Neurotransmitters and internal standards

Dopamine HCl, γ-aminobutyric acid (GABA), (-) epinephrine, (-), 2-phenylethylamine, serotonin HCl, and L-tryptophan were obtained from Sigma. 2-Phenyl-d_5-ethylamine, 4-aminobutyric-4,4-d_2 acid, 2-(3,4-dihydroxyphenyl)ethyl-1,1,2,2-d_4-amine HCl, epinephrine-2,5,6,α,β,β-d_6, *L*-tryptophan-2,3,3-d_3 and serotonin- α,α,β,β-d_4 creatinine sulphate complex, all purchased from CDN isotopes (Quebec, Canada) were used as internal standards for quantification. Stock solutions of all NT analytes and IS were prepared as 5 mM solutions in 50 % H_2O (HPLC grade) and 50 % 0.1 M HCl (Sigma) and stored at -80° C.

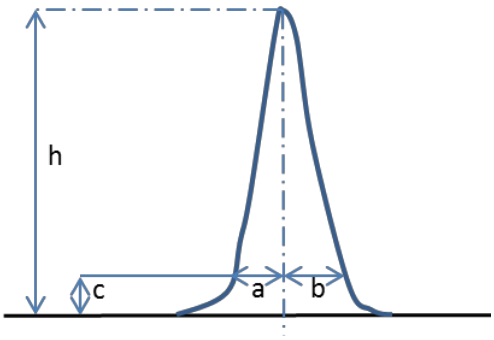
Figures



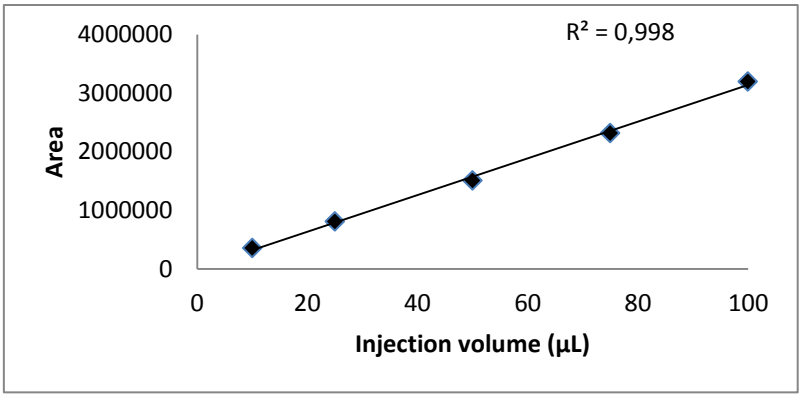
Supplementary Figure S1: **Illustration of the AFFL-SPE-cLC-MS platform.** Pump 1 is connected to the autosampler and transfers the injected sample to the 10 ports valve where the filter and the SPE column are located. When the valve is in position 1 (load) as illustrated here, the sample is loaded onto the SPE while the solvent goes directly to waste. When the valve is switched to position 2 (inject), showed in the figure above the system, pump 2 elutes the analytes off the SPE and onto the analytical column which is situated in a column oven set to 30° C. After being separated on the HILIC column the anlytes are transferred to the ESI where they are ionized before they enter the Orbitrap MS where they are detected by their *m/z* values and MS/MS fragmentations.

$$As = \frac{b}{a}$$

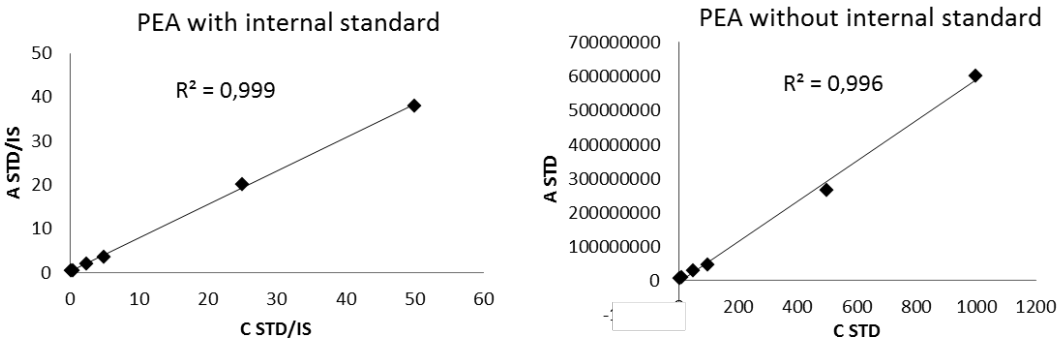
$$c = 0.1\,h$$



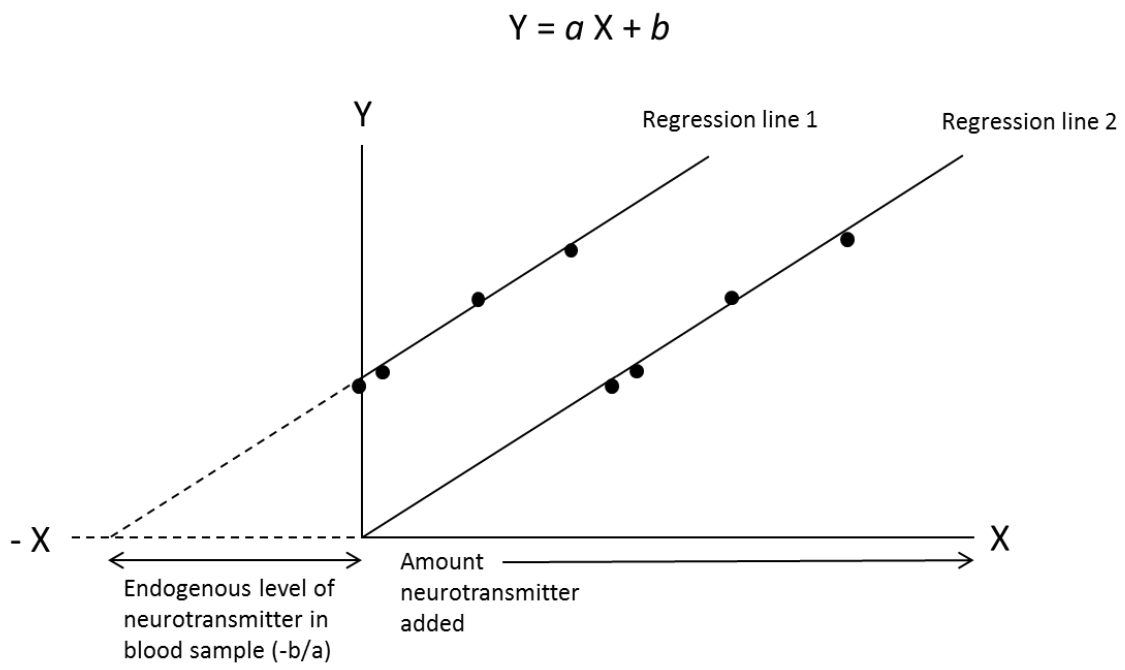
Supplementary Figure S2: Illustration of the method used for calculating the asymmetry factor (As).



Supplementary Figure S3: Plot of peak area vs injection volume. The injection volume was investigated from 10 to 100 µL (max injection volume of the auto sampler), by plotting the peak areas against the injection volumes. This gave a linear response with $r^2 = 0.998$, showing that no breakthrough occurred for these volumes.



Supplementary Figure S4: Calibration curves with and without internal standard. To see if it was possible to obtain trustworthy quantifications without using internal standards, calibration curves were constructed based on only the NT concentrations and NT peak areas. The R^2 value was still better than 0.99 for all NT analytes, when no internal standard was used, exemplified with calibration curves constructed for PEA *with* internal standard (left) and *without* internal standard (right).



Supplementary Figure S5: Calculation of NT concentrations. Illustration of the method used for correcting for the endogenous levels of NTs in blood. Regression line 1 is the original line, and regression line 2 is the corrected one.

Tables

Supplementary Table S1: **MS parameters**.

Parameters tune file		**Parameters MS/MS file**	
Sheat gas flow rate	5	Resolution	70 000
Aux gas flow rate	0	AGC target	1e5
Sweep gas flow rate	0	Max injection time	200 ms
Spray voltage (kV)	2.5	Isolation width	1 m/z
Capillary temperature	240	Fragmentation energy (NCE)	25 %
S-lens RF level	50		
Aux gas heater temperature	0		

Supplementary Table S2: **NTs identified/measured in whole blood samples.** Six NTs were quantified in whole blood samples from two volunteers. The results were compared to results obtained in other studies. Some of the differences can possibly be explained by the fact that in the other studies all of the NTs were quantified in plasma, not whole blood (WB), except for serotonin which was quantified in both plasma and whole blood, but the values varied a lot from study to study.

	Sample 1	**Sample 2**	**Other studies**
GABA	109 nM	80 nM	122 nM[1]
PEA	13 nM	13 nM	931 pg/mL (~8 nM)[2], 1130 pg/mL (~9 nM)[3]
Dopamine	< 1 nM	< 1 nM	0.01-0.35 nM[4]
Serotonin	83 nM	76 nM	0.36 nM[5], 4.8 nM (713 nM in WB)[6]
Adrenaline	0.8 nM	0.4 nM	0.01-1.3 nM[4]
Tryptophan	4 µM	4 µM	~1 µg/mL (~5 µM)[7]

Supplementary Table S3: Concentrations of the individual NTs in the STD mix and the internal standard mix. For the method development stock solution mixtures (STD mix) were made once a month and stored at -20° C. The same was done for internal standards (IS mix). Fresh working solutions were made daily by diluting the stock solution mixtures with ACN/H_2O (70/30) to appropriate concentrations. For validation, new stock solution mixtures with all compounds were made. These mixtures were stored at -80° C, and diluted to appropriate concentrations same day as the analysis.

	GABA	**PEA**	**Dopamine**	**Serotonin**	**Adrenaline**	**Tryptophan**
STD MIX (µM)	50	50	0.5	50	0.5	2500
IS MIX (µM)	20	20	10	15	10	300

Supplementary Table S4: Concentration levels used in validation and calibration samples. The six concentration levels of the NTs used in the validation and calibration samples given in nM, and the constant concentrations of the internal standards in all validation and calibration samples. Since the expected levels of endogenous NTs varied from pM to µM, each concentration level (XL-XH) contained individual concentrations of the different NTs. The concentration levels of internal standards were adjusted according to this, but also after their individual signal intensities, to ensure that all internal standards could be easily quantified.

	Concentrations levels (nM)							
Neurotransmitter	**XL**	**L**	**ML**	**M**	**HM**	**H**	**XH**	**Internal standard**
GABA	5	10	50	100	500	1000	5000	20
PEA	5	10	50	100	500	1000	5000	20
Dopamine	0.05	0.1	0.5	1	5	10	50	10
Serotonin	5	10	50	100	500	1000	5000	15
Adrenaline	0.05	0.1	0.5	1	5	10	50	10
Tryptophan	250	500	2500	5000	25 000	50 000	250 000	300

Notes

Calculation of efficiency

Efficiency (N) was calculated using the formula:

$$N = 5.54 \times \left(\frac{t_R}{t_{w_{0.5}}}\right)^2$$

t_R is the retention time of the analyte, and $t_{W0.5}$ is the width at half of the peak height.

Mobile phases

70% ACN and 30 % 100 mM ammonium acetate: the more polar compounds (e.g. dopamine) were not eluted off the column. Poor peak shape were obtained for the other NTs.

70 % ACN and 30 % 50 mM ammonium formate: irreproducible results with severe band broadening and sometimes no elution of the more polar compounds. No significant decrease in background noise was observed.

75 % ACN and 25 % 120 mM ammonium formate: band broadening was observed for GABA and the efficiency went from 3400 (70 % ACN) to 2500, a 26 % decrease.

80% ACN and 20 % 150 mM ammonium formate: peaks were broad and irregular, so N could not be measured properly.

Online oxidation

Preliminary studies were done on an Esquire 3000+ Ion trap MS (Bruker Daltonics, Billerica, MA, USA). Adding HCl to the NT stock solutions and having an acidic pH in the mobile phase were sufficient to prevent NT oxidation when this instrument was used. The method was moved to an Orbitrap MS when the need for better resolution and sensitivity arise, but severe oxidation (80-90%) of the catecholamines (dopamine and adrenaline) was then observed. When the flow was increased from 4 µL/min to 8 µL/min the oxidation decreased significantly, and it was assumed that the oxidation happened online during the analysis. It was also assumed that the ESI source was involved in the oxidation, since the configuration of this was quite different between the Orbitrap and ion trap MS. In the Orbitrap MS the voltage is on the emitter, and to protect the operator and the upstream equipment from being exposed to high voltage, a grounded contact is often placed upstream of the emitter electrode. But then a second upstream circuit is added and electrochemical reactions can occur[8]. In most cases, and for most analytes, this is not a problem, but when the analytes are easily oxidized (like the catecholamines), the mobile phase has a high conductivity (e.g. high amounts of salt) and the flow rate is relatively low, electrochemical reactions can occur. To avoid oxidation, 300 µM ascorbic acid was added to the mobile phase to act as an antioxidant [9]. No more oxidation was observed, and there were no interferences from the ascorbic acid even though it had the same *m*/*z* value as serotonin (177). Since ascorbic acid is light sensitive, the mobile phase bottles were covered with alumina foil, and new mobile phases were made daily during the method validation and sample analyses.

SPE column materials

Porous graphitic carbon (Hypercarb): None of the NTs were retained.

Strong cation exchange (SCX): All NTs were retained, but to avoid severe band broadening, 30 % water had to be used in the loading mobile phase. Hence a water plug was eluted off the SPE and onto the analytical column and this resulted in a loss of separation. And excessive amount of interferences from blood were also retained by the SCX material and subsequently released to the analytical column.

Weak cation exchange (WCX): All NTs were retained, but the same problem occurred as with the SCX. The recovery was also slightly lower.

Calculation of cLOD

The determination of the concentration limit of detection (cLOD) was limited by the lack of blood without NTs (blank matrix). Using standard samples instead would give an irrelevant cLOD since the matrix effects in the blood would not be accounted for. A crude estimate was therefore calculated for each NT by measuring the noise in the baseline close to the analyte peak in the MS/MS chromatograms from a pooled blood sample. Then the calibration curves from the validation were used to estimate the expected concentration at a signal to noise ratio of 3 (s/n = 3) in blood.

Precipitants

Four different organic solvents were evaluated as precipitant; ACN, isopropanol, methanol and ethanol. Even though isopropanol gave the best recovery, ACN was chosen since there would be no need for evaporating and resolving the sample prior to HILIC chromatography, which is time consuming and can contribute to loss of analyte.

Calculation of NT concentrations

Peaks were manually integrated using the XCalibur software, and neurotransmitter concentrations were calculated with Excel using the formula “y = a x + b”, equalling:

$$\frac{C_{NT}}{C_{IS}} = a \times \frac{A_{NT}}{A_{IS}} + b\frac{CNT}{CIS}$$

C_{NT} is the concentration of the NT of interest, C_{IS} is the concentration of the IS, A_{NT} is the area of the NT peak, while A_{IS} is the area of the peak corresponding to the IS. a is the slope of the regression line and b is the intercept with the y-axis. To correct for the endogenous concentrations of NTs in the calibration samples, $C\ NT/C\ IS$ was set to 0, and then A_{NT}/A_{IS} would correspond to $-b/a$. By adding this values ($-b/a$) to all X values, the regression line was moved on the positive side of the plot and the new regression equation, with $b \sim 0$, was used for calculating the concentrations of NTs in blood samples.

1 Bjork, J. M. *et al.* Plasma GABA levels correlate with aggressiveness in relatives of patients with unipolar depressive disorder. *Psychiatry Res.* **101**, 131-136 (2001).

2 Zhou, G., Miura, Y., Shoji, H., Yamada, S. & Matsuishi, T. Platelet monoamine oxidase B and plasma β-phenylethylamine in Parkinson's disease. *J. Neurol., Neurosurg. Psychiatry* **70**, 229-231 (2001).

3 Kawamura, K. *et al.* Improved method for determination of β-phenylethylamine in human plasma by solid-phase extraction and high-performance liquid chromatography with fluorescence detection. *J. Liq. Chromatogr. Related Technol.* **23**, 1981-1993 (2000).

4 Peaston, R. T. & Weinkove, C. Measurement of catecholamines and their metabolites. *Ann. Clin. Biochem.* **41**, 17-38 (2004).

5 Anderson, G. M., Hertzig, M. E. & McBride, P. Brief report: platelet-poor plasma serotonin in autism. *J. Autism Dev. Disord.* **42**, 1510-1514 (2012).

6 Hara, K., Hirowatari, Y., Shimura, Y. & Takahashi, H. Serotonin levels in platelet-poor plasma and whole blood in people with type 2 diabetes with chronic kidney disease. *Diabetes research and clinical practice* **94**, 167-171 (2011).

7 Bjork, J. M., Dougherty, D. M., Moeller, F. G. & Swann, A. C. Differential behavioral effects of plasma tryptophan depletion and loading in aggressive and nonaggressive men. *Neuropsychopharmacology* **22**, 357-369 (2000).

8 Kertesz, G. J. V. B. & Vilmos. Using the electrochemistry of the electrospray ion source. *Anal. Chem.* **79**, 5510-5520 (2007).

9 Plattner, S., Erb, R., Chervet, J.-P. & Oberacher, H. Ascorbic acid for homogenous redox buffering in electrospray ionization–mass spectrometry. *Anal. Bioanal. Chem.* **404**, 1571-1579 (2012).