Comprehensive analysis of nine monoamines and metabolites in small amounts of peripheral murine (C57Bl/6J) tissues

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ABSTRACT

Monoamines, acting as hormones and neurotransmitters, play a critical role in multiple physiological processes ranging from cognitive function and mood to sympathetic nervous system activity, fight-or-flight response, or glucose homeostasis. In addition to brain and blood, monoamines are abundant in several tissues, and dysfunction in their synthesis or signaling is associated with various pathological conditions. It was our goal to develop a method to detect these compounds in peripheral murine tissues.

In this study, we employed a high performance liquid chromatography method using electrochemical detection that allows not only detecting the catecholamines but a detailed analysis of nine monoamines and metabolites in murine tissues. Simple tissue extraction procedures were optimized for muscle (gastrocnemius, extensor digitorum longus, and soleus), liver, pancreas, and white adipose tissue in the range of weight between 10¬200 mg.

The system allowed a limit of detection between 0.625 pg µL-1 and 2.5 pg µL-1 for monoamine analytes and their metabolites, including dopamine, 3,4-dihydroxyphenylacetic acid, 3-methoxytyramine, homovanillic acid, norepinephrine, epinephrine, 3-methoxy-4-hydroxyphenylglycol, serotonin, and 5-hydroxyindoleacetic acid. Typical concentrations for different monoamines and their metabolization products in these tissues are presented for C57Bl/6J mice fed a high fat diet.

Keywords: high performance liquid chromatography; electrochemical detection; ECD; neurotransmitters; peripheral tissues;

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1. INTRODUCTION

Monoamines (MAs), including epinephrine (E), norepinephrine (NE), dopamine (DA), and serotonin (5-HT), play a critical role in the functioning of several living organisms. They are abundant in plants (Kulma and Szopa 2007), small crustaceans (Pfister, Rieb, Avramov, Rock, Griebler and Schramm 2013), and mammals (Palkovits and Brownstein 1989). In the brain, MAs act as neurotransmitters and control behavior such as motivation (Morita, Morishima, Sakai and Kawaguchi 2013), aggression (Volavka, Bilder and Nolan 2004), and appetite (Jason 2001). They are further present in other compartments of the body wherein they act as hormones. 5-HT is most abundant in the gut where it was thought to regulate gastrointestinal function, but its exact role is not yet fully understood.(Bornstein 2012) Other well-known hormones are E and NE, which are involved in the acute stress response of the body.(Charmandari, Tsigos and Chrousos 2004) Imbalances of MAs can result in several dysfunctions. At the level of the central nervous system (CNS), MA abnormalities have been implicated in cognitive and mood disorders, as well as in motor disabilities. (McCann, Penetar, Shaham, Thome, Sing, Thomas, Gillin and Belenky 1993, Vernier, Moret, Callier, Snapyan, Wersinger and Sidhu 2004) In peripheral organs and tissues, high levels of circulating MAs seem to be associated with cardiac hypertrophy, heart failure, and hypertension.(Dominiak and Grobecker 1982, Scheuer 1999)

MAs are built from amino acids and can be transformed into various metabolites (Figure 1). From a chemical point of view, MAs and their metabolites are electroactive compounds and can, therefore, easily be quantified using an electrochemical detector (ECD). This technique is in general considered cheap, highly selective, and sensitive. Eisenhofer *et al.* describe the advantages and disadvantages of ECD and MS system. (Eisenhofer, Peitzsch and McWhinney 2016) MS is able to detect more analytes, however, in our laboratory the MS system could not achieve a high sensitivity for the catecholamines and a derivatization process would be needed for the analysis. (Pfister, Rieb, Avramov, Rock, Griebler and Schramm 2013). ECD is also much more specific for the monoamines than MS detectors. Therefore, although in the last decades several other detectors have been tested for the quantification of such derivatives, high performance liquid chromatography (HPLC) with electrochemical detection is still considered one of the best choices, indicated by the high number of reports. (Allen, Rednour, Shepard and Pond , Bidel, Corvaisier, Jozet-Alves, Pottier, Dauphin, Naud and Bellanger 2016, Birbeck and Mathews 2013, Ferry, Gifu, Sandu, Denoroy and Parrot 2014, Ganesana, Lee, Wang and Venton 2017, Nguyen, Aerts, Van Dam and De Deyn 2010, Parrot, Neuzeret and Denoroy 2011, Schou-Pedersen, Hansen, Tveden-Nyborg and Lykkesfeldt 2016, Smith, Schwartz and Lucot 2013, Tsunoda 2006)

In clinical diagnostics, HPLC–ECD is routinely used for MA detection in human blood, plasma and urine, while in the academic environment, this technique is most often applied to the analysis of MAs in animal brains. However, little focus has been directed toward accurately detecting MA levels in peripheral tissues. Mano *et al.* reported using ECD for measuring MAs in rat heart muscle.(Mano, Sakamoto, Fujita, Makino, Kakizawa, Nagata, Kotake, Hamada, Uchimura, Hayakawa, Hayashi, Nakai, Itoh, Kuzuya and Nagasaka 1998) However, they had to use a gradient system equipped with 16 ECD sensors for the detection of NE, E, DA, 5-HT, and several MA metabolites. Since standard detectors only have one or two ECD cells, the method by Mano *et al.* appears difficult to apply in regular laboratory settings. Eldrup *et al.* used a method based on alumina extraction, which restricted MA detection in rat tissues including muscles to the catecholamines NE, DOPA, and DA due to their selective binding properties.(Eldrup, Richter and Christensen 1989) For the pancreas, four studies using HPLC–

ECD were reported: two analyzed tissue and two others analyzed perfusates or islets.(Adeghate, Ponery and Sheen 2001, Lundquist, Aléh, Hansson and Håkanson 1989, ÖStenson, Hjemdahl and Efendic 1993, Yi, Smith, Baker and Love 2004) The studies on tissues were performed in rats on NE, E, and 5-HIAA and in rabbits on the catecholamines and 5-HT. The studies for perfusate and islets both used the alumina method and the same restrictions in MA detection as above applied. Similarly, Garofalo *et al.* reported MA detection in white fat of rats via an alumina–based method.(Garofalo, Kettelhut, Roselino and Migliorini 1996) Lastly, Huang *et al.* showed a measurement of catecholamines in murine liver; however the compounds were detected via UV after chemical derivatization.(Huang, Guo, Wang and Zhang 2014) Recently, Yang *et al.* claimed a new methodology for the simultaneous analysis of seven MAs in a variety of biological samples including peripheral tissues, using a conventional HPLC system. (Yang and Beal 2011) However, only data about bioamine levels in mouse striatum were reported. A recent paper reported the determination of 11 compounds in a single run, but it was only applied to rat hippocampus and cortex.(Zhang, Yang, Luo, Shang and Jiang 2016) In practice, the simultaneous measurement of monoamines and their metabolites in peripheral tissue using a common electrochemical detector was limited in the number of compounds and not applied to mice. Thus, the monitoring of several monoamines together with their metabolites in different tissues is somehow a missing aspect in this field. The quantification of monoamines with their major metabolites in a single run can give new insight into the biosynthetic and metabolic pathway of such derivatives and therefore can open the way to a more comprehensive overview of physiopathology of diseases. This can permit not only deeper knowledge of the disorder affecting patients but may also pave the way to a better understanding of the therapy to apply.

However, it is important to mention that before going to humans, a robust preclinical study is required. In general, a large part of animal models are based on mice and the most common genetic background is the so called C57Bl/6J. For that reason, we developed our method in this mice strain. Furthermore, we tried to develop a method in which the least amount of sample tissue was required, so that the methodology can be applied to analysis of MAs in specific organ regions, i.e. left and right ventricle.

In summary, in this paper we describe a new analytical method for the simultaneous quantification of nine monoamines (dopamine, 3,4-dihydroxyphenylacetic acid, 3-methoxytyramine, homovanillic acid, norepinephrine, epinephrine, 3-methoxy-4-hydroxyphenylglycol, serotonin, and 5-hydroxyindoleacetic acid) in mice tissues using a HPLC-ECD system. The tissues comprise liver, pancreas, muscles (gastrocnemius, soleus, extensor digitorum longus), and white adipose tissue. For all analyzed tissues, we developed an easy and fast clean-up (20-30 min) and a short analytical analysis (15 min). The method is usable for high throughput and can be applied in preclinical and clinical studies. To the best of our knowledge, this is the first report to simultaneously analyze nine different MAs in mice tissue different from the brain, using a standard HPLC-ECD system.

2. EXPERIMENTAL

2.1. Chemicals and reagents 3-Methoxy-4-hydroxyphenylglycol (MHPG) hemipiperazinium salt, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 3-methoxytyramine (3-MT) hydrochloride, 5-HT hydrochloride, 5-hydroxyindoleacetic acid (5-HIAA), and perchloric acid (HClO4, 70 %) were purchased from Sigma Aldrich. A catecholamine standard (1 mg/mL), containing NE, E, and DA, as well as a 3,4-dihydroxybenzylamine (DHBA) standard (1 mg/mL), used as the internal standard (IS), were obtained from Thermo Fisher Scientific. A commercially available Mobile Phase from the company RECIPE (Mobile Phase, Order No. 1210, ClinRep® commercial HPLC) was used during the experiments. Merck supplied Acetonitrile (ACN), applied as an organic modifier, and LC–MS grade water. All chemicals were acquired in the highest available purity and used without further purification.

2.2. Tissue collection and extraction All procedures involving animal handling were approved by the committee for Care and Use of Laboratory Animals of the Government of Upper Bavaria, Germany. Male high–fat diet fed C57Bl6J mice were sacrificed via cervical dislocation; the specified organs were dissected, immediately snap frozen in liquid nitrogen and stored at -80 °C until further processing.
Tissues were extracted in 1.5 mL tubes (Sarstedt, 72.706) to provide sufficient homogenization. The differential fat content in the tissues required specific clean-up procedures, which are summarized below.

2.2.1. Brain and Pancreas 200 µL of 0.3 M $HClO_4$ and 4 µL of DHBA (IS) were added to the brain (10–15 mg) or pancreas (130–200 mg), followed by homogenization via ultrasonication (Bandelin Electronics, UW-70) on ice for 30 s. The homogenate was centrifuged at 7879 g for 10 min at 4 °C. Afterwards, the supernatant was transferred into a sample vial (Supelco, P/N 29409-U) with a snap cap (Supelco, P/N 27427) and injected into the system.

2.2.2. Muscle All muscles (gastrocnemius (150–190 mg), soleus (10–20 mg), and extensor digitorum longus (EDL, 20–30 mg)) were treated in the same manner. The tissue was first powdered in a liquid N_2–cooled mortar and then stored again at -80 °C until further extraction. On the day of the measurement, 400 µL of 0.3 M $HClO_4$ for the gastrocnemius, 200 µL for the smaller muscles, and 4 µL of DHBA (IS) were added and the sample was homogenized by ultrasonication on ice for 2 × 30 s. The homogenate was centrifuged at 7879 g for 10 min, the supernatant transferred into a sample vial and injected into the system.

2.2.3. White Adipose Tissue Epididymal white adipose (eWAT, 100–120 mg) was homogenized in 200 µL of 0.3 M $HClO_4$ and 4 µL of DHBA (IS) by ultrasonication on ice for 30 s. The homogenates were centrifuged at 7879 g for 10 min. The solution was collected with a 1 mL syringe and a cannula without disrupting the top fat layer. To remove traces of fat, it was subjected through a 0.2 µm filter (Whatman, P/N 6784-0402). A small pocket of air was collected prior to piercing the pellet to facilitate pushing the entire small volume of solution through the filter. The filtrate was collected in a sample vial and injected into the system.

2.2.4. Liver After addition of 400 µL $HClO_4$ and 4 µL DHBA (IS) to the liver (130–200 mg), it was homogenized for 30 s by ultrasonication on ice. The resulting homogenate was centrifuged at 7879 g for 10 min, the supernatant transferred into a sample vial and injected into the system.

2.3. Analytical instrumentation and chromatographic conditions Measurements were conducted on a HPLC–ECD system from Dionex. The system comprised a gradient pump (GP50), a cooled autosampler (AS50) with an automatic injection valve, a 25 µL sample loop, and an electrochemical detector (ED50A) with one flow cell with a glassy carbon working electrode (Thermo Scientific, 044113).
The injection volume for all measurements was 20 µL. All the samples were injected once.
Separation of the compounds was carried out at a flow rate of 1 mL/min on a solid core particle reversed phase C18-column from Thermo Fisher Scientific (Accucore XL C18, 150 × 4.6 mm, 4 µm). A security cartridge of the same material (Thermo Scientific, P/N 74104-014001) prevented blocking of the column from residues. The column oven was set to 40 °C.
For measurements, an isocratic elution with a commercially available mobile phase from RECIPE with 0.25 % v/v ACN was used. The pH value was lowered to 3.85 ± 0.01 using HClO4 (70%) before addition of the organic modifier. The mobile phase was degassed under vacuum in an ultrasonic bath (Bandelin, Sonorex RK 156) prior to use.
The potential of the detector was set to 0.7 V against an Ag/AgCl reference electrode (Thermo Scientific, P/N 044198). The sensitivity display range was 10 nA and the data transfer rate to the computer 10 Hz. The Chromeleon 6.5 (Thermo Fisher) software was applied to control the system and to process the chromatograms.

2.4. Recovery To account for the loss of analytes during sample clean-up, an IS was added to the calibration solution and to each sample. The recovery was then calculated according to the formula:

$$R = \frac{Area\ IS_{sample} \cdot Amount\ IS_{\ calibration}}{Area\ IS_{calibration} \cdot Amount\ IS_{\ sample}}$$

The Chromeleon software automatically adjusts the values for the analytes, according to the recovery of the internal standard.

2.5. Standards A standard solution at a concentration of 1 µg µL-1 was produced by dissolving 10 mg DOPAC, 12.2 mg 3-MT hydrochloride, 10 mg HVA, 12.35 mg MHPG hemipiperazinium salt, 10 mg 5-HIAA, and 12.1 mg 5-HT hydrochloride in 10 mL of 0.3 M HClO4. Standard solutions of the catecholamines NE, E, and DA as well as of the internal standard DHBA at the same concentrations were commercially available.
To produce an internal standard solution of 1 ng µL-1, 100 µL of the original standard was diluted with 100 mL of 0.3 M HClO4. To prepare the working standard solution of the analytes, 100 µL of the catecholamine standard and 100 µL of the previously prepared monoamine standard were diluted in 100 mL of 0.3 M HClO4 in the same flask.
For operational ease, 200 µL of both the 1ng µL-1 IS and analyte standard solution were diluted in 3.6 mL of 0.3M HClO4 to prepare a working standard of 1 ng 20 µL-1 for the calibration, as 20 µL was the injection volume of the system. This was stored at -80 °C and remained stable for a month. After this time, a new standard was produced. No antioxidant was used as this could cause disturbances in the early part of the chromatogram and the standard was stable without it (Supplemental 2.).

2.6. Method Validation

To determine the linearity of detection, standard solutions within the expected tissue concentrations were injected into the system in triplicates and plotted in a graph by the Chromeleon Software. The R^2 value was calculated from the trend line, and $R^2 > 0.99$ was deemed acceptable. The concentrations injected were 20, 10, 5, 1, 0.5, and 0.1 ng 20 µL-1.

The limit of detection (LOD), defined as a signal higher than three times the height of the base line noise, was measured by diluting a standard solution (1 ng 20 µL-1 per analyte). The height of the baseline was determined by injection of a LC–MS grade water blank.

The limit of quantification (LOQ) is generally defined as 10 times the height of the base line noise. Values were determined during the LOD measurements.

The intra- and interday precision were determined in standard solution and in spiked samples to account for differences due to matrix effects. For the standard measurement of the intraday repeatability, a standard solution (1 ng 20 µL-1 for each analyte) was injected five times. Interday repeatability was measured by injecting a standard solution (1 ng 20 µL-1 for each analyte) once on five days over two weeks. Standard deviations were calculated for each compound. To determine the intra- and interday precision in biological samples, eWAT, gastrocnemius, and hypothalamus were chosen. eWAT and gastrocnemius are on the opposite spectrum of fat and protein content, and the hypothalamus contains the highest concentration of analytes. Samples were homogenized and aliquoted into blank and spiked samples with 0.5 ng, 1 ng, and 5 ng of analytes per injection. They were then extracted and injected into the system in triplicates. The interday precision was measured three times over 8 days.

To measure the accuracy, the percentage of recovery of the spiked analytes was calculated from the spiked results minus the blank endogenous values.

For confirmation of selectivity, a complete blank of $HClO_4$ was put through the extraction procedure, injected into the system, and analyzed for any peaks.

3. SAFETY CONSIDERATIONS

$HClO_4$ (H226, H314, H402; P210, P233, P240, P241, P242, P243, P260, P264, P273, P280, P301/330/331, P303/353/361, P304/340, P305/351/338, P310, P363, P370/378, P403/235) is a very strong acid that etches strongly and should be handled with great care. It is highly corrosive and contact with metal should be avoided. It can form explosive peroxides and should be kept in the dark to prevent peroxide formation.

ACN (H225, H302, H312, H319, H332; P210, P280, P305/351/338) can be metabolized to hydrogen cyanide, which is a lethal gas that stops cellular respiration. ACN is possibly absorbed through the skin; therefore, any contamination should be avoided.

MAs have no risk and safety phrases; however they are endogenous compounds that have strong biological effects. Great care should be taken to prevent exposure orally or via mucous membranes.

The laboratory safety guidelines must be followed at all times and wearing personal protective equipment when handling the above mentioned substances is mandatory.

4. RESULTS AND DISCUSSION

4.1. Analysis Time

Solid Core particle columns are becoming more popular, as they result in increased separation efficacy and lower back pressure, compared to fully porous particles. Adoption of the Thermo Fisher solid core particle Accucore column allowed to increase the flow rate to 1 mL/min at 120 bar, which would previously have overreached the pressure limit of 260 bar with a conventional C18-column. This shortened the run time by more than half from 40 min with the previous method (Supplemental 3.) to 13 min with the current method. This is between the time of two recent methods developed by Allen *et al.* and Zhang *et al.* (Allen, Rednour, Shepard and Pond , Zhang, Yang, Luo, Shang and Jiang 2016). Figure 2 shows a chromatogram of a standard solution (Retention times shown in Supplemental 1.).

4.2. Method Validation

The detector was linear in the range between 0.1 ng 20 µL-1 and 20 ng 20 µL-1. This covers the concentration range expected in biological samples. The correlation factors, as calculated by the Chromeleon software, were above the accepted value of 0.99 for all the analytes.

LOD and LOQ were determined for each analyte. The LOD ranged from 0.625 pg/µL to 2.5 pg/µL, while the LOQ was found to be between 2.5 pg/µL and 5 pg/µL (Table 1). These values are in the same range or lower than Zhang *et al.* achieved with a boron doped electrode, but four to five fold higher than the ones found by Allen *et al.*

Intraday and interday precision for standards and biological samples was within the limits of the US Food and Drug Administration's requirements for bioanalytical method validation. The precision of the standards is reported in Table 2 and was below 10 %. The precision in biological samples is shown in Table 3. It was below 5 % for all the analytes and in the same range as values found by Zhang *et al*.

Accuracy was calculated as the recovery of the spiked analytes. Deviations in biological samples were less than 15 % for all analytes and generally below 10 %, as suggested by the US Food and Drug Administration's requirements for bioanalytical method validation (Table 3). These values are also in accordance with Zhang *et al.* and Allen *et al.* The only exception to this is MHPG. In biological samples, MHPG is eluting early and partly hidden in an interference peak, and can therefore not be quantified accurately. We still included it in our results, as it is still visible and might be usable in a direct comparison between WT and KO models, where the accurate absolute concentration is not as important as the differences between different mice, which might still be detectable semi-quantitatively. The chromatogram of the blank solvent subjected to the clean-up contains no peaks (Figure 3 i). This is proof of the selectivity of the method.

For the extraction procedure, it was advisable to only use a 1.5 mL–sized Eppendorf tube as higher volume tubes come with a leveled lower end, which allows too much space and was insufficient for homogenization using ultrasonication, particularly for muscle tissues. IS recovery in the samples varied between 0.5 and 0.95, but averaged at 0.7-0.8. This shows that the homogenization and extraction procedures were good and consistent.

The validated method is linear in the expected concentration range, is selective, and is below the precision and accuracy requirements published by the FDA. The sensitivity is within the range found in two recent publications.

4.3. Concentrations of analytes in different tissues MAs were detected in all the tested murine tissues. NE, its metabolite MHPG, and 5-HT were measured in all the samples, although the content varied significantly between tissues (Table 4). Other MAs were partially detected, but the concentrations and ratios varied between tissues. All of the analytes were identified with spiking experiments to the real samples. Figure 3 shows the chromatograms of the biological samples. There are multiple unknowns, especially in the front of the chromatograms, but except for MHPG the known analytes are not affected.

In the research of wildtype (WT) and knockout (KO) models, a comparison of MAs might reveal not only differences in absolute values, but variances might be hidden in the ratios between analytes. For example, changes in the ratios between a compound and its metabolites may indicate the activation or inhibition of degradation pathways.(Gogos, Morgan, Luine, Santha, Ogawa, Pfaff and Karayiorgou 1998) Moreover, changes in the ratios between neurotransmitters can be the result from an excess of external stressors and might affect physiological processes.(Leite, Rodrigues, Soares, Marubayashi and Coimbra 2010) Some examples for the interpretation of ratios are given. Supplemental 4. gives an overview over the ratios between active compounds and metabolic products as well as ratios between active compounds.

4.3.1. Hypothalamus The hypothalamus is a small part of the brain that controls numerous physiological functions e.g., energy balance (Dietrich and Horvath 2013) and arousal (Adamantidis and de Lecea 2008). Therefore, it is often regarded as the homeostasis center of the brain. In this study, the hypothalamus was the only brain region analyzed. In a previous study of the same mouse model, however, other regions, namely the prefrontal cortex, olfactory bulbus, midbrain, striatum, hippocampus, and even amygdala were also analyzed using the same extraction method (but slightly different HPLC system). Accordingly, values found in the hypothalamus allow for direct comparison with the previous study (Supplemental 3.). Hypothalamic concentrations were the highest for most MAs across all tissues. This was not surprising, as the brain comprises mostly neurons releasing neurotransmitters. Only the MHPG amounts in the pancreas were slightly higher than that in the brain. Notably, the amounts of MA metabolites in the hypothalamus were lower than the amount of the parent MAs; whereas for peripheral tissues, it was the other way round. The only exception was the pair 5-HT/5-HIAA wherein the metabolite was lower in both the hypothalamus and peripheral tissues. In the hypothalamus, the calculated ratio between DA:HVA was 5.8, while for NE and 5-HT, the ratios to their metabolites (MHPG and 5-HIAA) were 2.2 and 2.4, respectively. This indicated that most of the active compound is reabsorbed by the synapses and restored into vesicles wherein the metabolic enzymes cannot access and metabolize them.(Rudnick and Clark 1993) Another example for the use of ratios is the two DA metabolic pathways. One pathway starts with the enzyme MAO and leads, via one intermediate product and DOPAC, to the end product, HVA. The other pathway starts with the enzyme COMT and results, via 3-methoxytyramine and another intermediate, in HVA as well (Figure 1). COMT is mostly found in the synapses, whereas MAO is mostly found in the presynaptic terminal.(Phillis 1970) The fact that DOPAC, but no 3-MT, was detected indicates that the initial metabolizing of DA takes place after the reuptake in the presynaptic terminal and that the other pathway is inactive. The ratios NE:5-HT (1.3), DA:NE (0.4), and DA:5-HT (0.5) indicate that the innervation with NE neurons was the highest in the hypothalamus, followed by serotonergic neurons. Dopaminergic neurons seem to have had the lowest innervation.

4.3.2. Muscle

EDL The EDL is a muscle on the anterior part of the lower leg. It is responsible for dorsiflexion of the foot and comprises mainly fast twitch type II muscle fibers.(Soukup, Zachařová and Smerdu 2002) Concentrations of all the analytes in the EDL were much lower compared to the hypothalamus and reflected the fact that less neurons innervate the muscles.(Peyronnard, Charron, Lavoie and Messier 1986) The presence of DOPAC and HVA in the EDL indicated that DA was also present at some point and was below the LOD. DA is mostly associated with the central nervous system; yet, there is also evidence that it may play a role in the peripheral system.(Rubí and Maechler 2010) However, given the low amount of DA metabolites and lack of parent compound, innervation of dopaminergic neurons seems unlikely. Residual DA and metabolites may be present as side products in the formation of NE, which is approximately 90% efficient.(Eisenhofer, Friberg, Rundqvist, Quyyumi, Lambert, Kaye, Kopin, Goldstein and Esler 1996) E was below the LOD, as it is only released into the bloodstream via the adrenal medulla.(Bohlen 2006) The ratio for 5-HT and 5-HIAA could not be calculated, because the metabolite was not found in the EDL. This goes against the catecholamine's trend wherein the metabolites are higher than the parent compound in peripheral tissues. However, 90 % of 5-HT in the body is produced in the gastrointestinal tract, stored by cells with a 5-HT transporter and released as necessary.(Ruddell, Mann and Ramm 2008) Therefore, after the release, the active compound can be either reabsorbed or transported away to the liver wherein it is metabolized to 5-HIAA, which is then excreted via the urine.(Tyce 1990) The ratio for NE:5-HT was 2.0 and therefore higher than in the hypothalamus. This ratio was also the highest of all three muscles and possibly reflects the content of the fast twitch muscle fibers. 5-HT, as an inhibitory agent, reduces twitch tension; therefore it could be lower here.(Takamori 1977)

Soleus The soleus is a muscle on the posterior part of the lower leg. Its function is the plantarflexion of the foot and as it is very important for walking, it contains mostly slow muscle fibers. In rats, it comprises almost 100 % slow twitch type I fibers.(Soukup, Zachařová and Smerdu 2002) It was the smallest muscle analyzed. The amounts of MAs in the soleus were far lower than in the hypothalamus. However, the values were higher than the ones found in the EDL. The metabolites of DA were about doubled, which also applied for NE. Four times the amount of MHPG was found and 5-HT was tripled. Possibly, a difference exists in the use of MAs for transmission of signals between slow and fast twitch muscle fibers. The presence of the metabolites DOPAC and HVA also indicated the former presence of DA. The ratio between NE/5-HT was 1.3, which was the lowest of the three muscles. It seems that in the slow twitch fibers more 5-HT is present, probably to control the tension and facilitate exertion. The use of the soleus for longer exertion might also explain the highest amount of MAs compared to the other muscles.

Gastrocnemius The gastrocnemius is a muscle of the posterior part of the lower leg and together with the soleus forms the calf. Its function is also the dorsiflexion of the foot, but as it contains a mix of slow twitch type I and fast twitch type II muscle fibers, it is mostly used for running.(Staron, Kraemer, Hikida, Fry, Murray and Campos 1999) It was the biggest muscle analyzed. The amounts of MAs in the gastrocnemius were lower than in the soleus and about the same as in the EDL. The only exceptions are NE, which was lower, and MHPG, which was higher than in the fast twitch muscle. 5-HIAA could only be detected in two samples. The gastrocnemius was the only muscle where DA could be detected. Moreover, the ratio between the two metabolites DOPAC:HVA (0.8) was much lower than in the other tissues analyzed, indicating a delay in the degradation of the neurotransmitter and therefore less DOPAC. The ratio between NE:5-HT, however, was at 1.4 and slightly higher than in the soleus. This might reflect the fact that the gastrocnemius is a mixture of type I and type II fibers.

4.3.3. eWAT eWAT is a fat pad near the testicles and important for spermatogenesis in rodents.(Chu, Huddleston, Clancy, Harris and Bartness 2010) eWAT had the lowest monoamine levels of all the tissues across all analytes. This might be due to the fact that white adipose tissue is not very metabolically active.(Labbé, Caron, Chechi, Laplante, Lecomte and Richard 2016) Moreover, low NE levels indicate that innervation via the sympathetic nervous system was low. DA and E were only visible in one sample each. 5-HIAA was present in all but one of the samples.

4.3.4. Pancreas The pancreas is an endocrine organ that plays a vital role in digestion and blood sugar regulation.(Cano, Soria, Martín and Rojas 2014) The pancreas had monoamine levels higher than the eWAT. NE and MHPG were higher than in the soleus and DA levels were always above the LOD. However, 5-HT was lower and its metabolite, 5-HIAA, was at a higher level than in the muscles. MHPG levels were highest in the pancreas compared to all other analyzed tissues. 5-HT appears to be more rapidly degraded than in all the other tissues, as the ratio to its metabolite was just 1.8. In contrast, the NE:5-HT ratio was very high at 9.47. This high ratio might reflect a receptor–independent action profile for 5-HT in the pancreas, which binds covalently to proteins, thus making it impossible for our method to detect 5-HT.(Paulmann, Grohmann, Voigt, Bert, Vowinckel, Bader, Skelin, Jevšek, Fink, Rupnik and Walther 2009) Nevertheless, the presence of 5-HIAA shows that some degradation occurred and not all of the neurotransmitter was protein bound or transported away for metabolization.

4.3.5. Liver The liver is one of the major organs in the body of mammals and plays an important role in detoxification and metabolic homeostasis.(Reinke and Asher 2016) The levels of MAs in the liver were comparable to levels in the eWAT. Only NE and 5-HT were at a higher level, similar to those found in the gastrocnemius. This reflected innervation of the liver with serotonergic and norepinephrinergic neurons, which play an important role in homeostatic liver control.(Cousineau, Goresky and Rose 1983, Ruddell, Mann and Ramm 2008) The very low concentrations of DA metabolites suggested that DA does not play a functional role in hepatic metabolism. Rather, DA appears to function as a precursor for NE. The NE:5-HT ratio was 0.7, demonstrating that the liver was the only tissue wherein 5-HT was at a higher concentration than NE. Accordingly, our data corroborate the purported role of 5-HT in liver function.(Ruddell, Mann and Ramm 2008) The presence of 5-HIAA points to the liver as the main tissue for metabolizing 5-HT.

5. Comparison

In comparison with our former study of the hypothalamus of the same mouse model, the amounts of DA, DOPAC, HVA, and NE were about twice as high. (Supplemental 3.) MHPG was not included in the former method, thus no comparison could be made. 5-HT and its metabolite 5-HIAA were in the same ranges. In the previous study, 3-MT and E could be detected in very low amounts. Despite these differences, the ratios discussed above were in the same range for both studies. If the differences were of a systematic nature, due to a change of systems and methods, 5-HT and 5-HIAA would also be doubled. If the differences were due to an error in the standard preparation procedure, most likely single compounds or all the compounds would be affected. However, only analytes that belonged to the same metabolic pathway were affected. All of this indicates that biological variability caused the differences. This could also be further accentuated by the smaller sample size in the current study.

Previously published values for the hypothalamus indicated lower levels in the same range as those reported in this study.(Huotari, Gogos, Karayiorgou, Koponen, Forsberg, Raasmaja, Hyttinen and Männistö 2002, Lorden and Oltmans 1977) For the EDL and soleus, no published values could be found. Mano *et al.* tested rat cardiac muscle, which precludes comparison to our values from skeletal muscle.(Mano, Sakamoto, Fujita, Makino, Kakizawa, Nagata, Kotake, Hamada, Uchimura, Hayakawa, Hayashi, Nakai, Itoh, Kuzuya and Nagasaka 1998) They could not find any DA and E in their samples, which was similar to this study. Eldrup *et al.* found lower levels for NE and DA in the gastrocnemius, pancreas and liver of rats but could not detect more analytes.(Eldrup, Richter and Christensen 1989) Garofalo *et al.* detected NE in the eWAT of rats within the same range as this work.(Garofalo, Kettelhut, Roselino and Migliorini 1996) In the liver of mice, Huang *et al.* found 100–fold higher NE levels than this study.(Huang, Guo, Wang and Zhang 2014) However, they reported equally high NE levels in the whole brain, which seems unlikely.

Together, our results are in agreement with the reported literature. When comparing studies about MA values in the peripheral tissues, it can be noted that our method provides an improved extraction and procedure, more detectable analytes, and faster analysis times.

6. CONCLUSION

Our method offers detection of MA neurotransmitters and their most important metabolites in small volumes of murine brain and peripheral tissues, namely, skeletal muscle, liver, white adipose, and pancreas tissues.

Our method is easy to use, has a simple and fast clean-up procedure, and a short run time, thereby allowing high sample throughput at reduced costs. Besides accurate detection of absolute values, which are often difficult to determine and verify in biological tissues, our method allows for direct comparison of monoamine ratios, or parent MAs and their respective metabolites, within or between tissues of WT and KO mice or mice undergoing surgical or pharmacological interventions. In conclusion, we provide a valuable tool for researchers to better understand the role of MAs in the etiology of systemic and CNS-driven diseases.

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AUTHOR CONTRIBUTIONS

Joachim Nagler analyzed and interpreted the data and co-wrote the manuscript. Sonja Schriever dissected the samples and performed the extractions. Dr. Pfluger co-conceptualized the project and helped in the interpretation of the data. Prof. Schramm co-conceptualized the project, supervised the interpretation of data, and co-wrote the manuscript. All authors have approved to the final version of the manuscript.

CONFLICT OF INTERESTS

There are no conflicting interest and external payments.

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Table 1. LOD/LOQ of MAs

Analyte	MHPG	DOPAC	NE	E	5-HIAA	HVA	DA	3-MT	5-HT
LOD [pg µL-1]	2.5	0.625	1.25	1.25	2.5	1.25	1.25	2.5	2.5
LOQ [pg µL-1]	5	2.5	5	5	5	2.5	2.5	5	5

Table 2. Intra and interday precision in standard solution at a concentration of 1 ng 20 µL^{-1}. Measurements were done five times.

Analayte	Intraday [pg]	RSD	Interday [pg]	RSD
MHPG	969	2.2	983	7.9
DOPAC	1053	3.0	962	5.2
NE	1056	3.1	971	5.8
E	1052	2.8	963	5.9
5-HIAA	1018	1.1	953	3.9
HVA	1056	2.9	961	5.6
DA	1049	2.7	951	5.8
3-MT	1047	3.5	972	6.9
5-HT	1019	1.0	986	3.8

Table 3: Combined Intra- and interday precision and accuracy in spiked samples of hypothalamus (HYP), eWAT, and gastrocnemius (GASTR). Measurements were done in triplicates on three days over a period of 8 days.

Analyte	Spiked amount [pg 20 µL-1]	Mean detected amount [pg 20 µL-1]			RSD			Recovery [%]		
		HYP	eWAT	GASTR	HYP	eWAT	GASTR	HYP	eWAT	GASTR
MHPG	500	505	410	286	2.2	3.4	4.1	100.9	82	57.2
	1000	886	691	557	1.2	3.6	1.5	88.6	69.1	55.7
	5000	4417	3803	3499	1.2	2.9	1.9	88.3	76.06	69.98
DOPAC	500	509	429	415	1	0.8	1.3	101.8	85.8	83
	1000	969	897	886	1.3	1.2	2.5	96.9	89.7	88.6
	5000	4504	4474	4407	0.6	2.2	1.6	90.1	89.48	88.14
NE	500	535	495	431	0.8	0.7	1.2	107.0	99	86.2
	1000	1034	1024	933	2	1.9	2.3	103.4	102.4	93.3
	5000	4758	4934	4648	0.4	1.3	2	95.2	98.68	92.96
E	500	511	459	469	1.1	0.5	2	102.2	91.8	93.8
	1000	1032	990	972	2.1	2	2.4	103.2	99	97.2
	5000	4881	4965	4820	1	1.5	1.9	97.6	99.3	96.4
5-HIAA	500	520	500	468	1.4	1.7	2.6	104.0	100	93.6
	1000	1068	1067	990	3.9	3.3	2.9	106.8	106.7	99
	5000	4821	5325	5262	1.4	1.7	2.9	96.4	106.5	105.24
HVA	500	521	455	482	1.3	1.9	1.6	104.2	91	96.4
	1000	948	844	927	1	2.3	2.8	94.8	84.4	92.7
	5000	4444	4165	4311	0.4	1.9	1.2	88.9	83.3	86.22
DA	500	493	502	456	1.5	0.9	2.2	98.6	100.4	91.2
	1000	988	1022	921	1.8	2.6	4.6	98.8	102.2	92.1
	5000	4687	4830	4656	0.5	1.6	2.5	93.7	96.6	93.12
3-MT	500	515	481	493	1.5	2	2.5	103.1	96.2	98.6
	1000	1007	1005	978	2.3	2	2.6	100.7	100.5	97.8

	5000	5143	5310	5105	0.5	1.8	2	102.9	106.2	102.1
5-HT	500	529	532	498	1.3	2.2	2.6	105.8	106.4	99.6
	1000	1022	1007	977	2.1	4.4	2.8	102.2	100.7	97.7
	5000	4977	5112	5025	1.4	1.7	2.3	99.5	102.24	100.5

Table 4. Concentrations of MA analytes in murine tissues of 25 week-old C57Bl/6J mice fed high–fat diet for 16 weeks

Analyte [pg/mg]	Hypothalamus [n=5]	EDL [n=6]	Soleus [n=6]	Gastroc. [n=6]	eWAT [n=6]	Pancreas [n=4]	Liver [n=6]
DA	1301 ± 313	n.d.	n.d.	6 ± 5	1 ± 3	29 ± 23	n.d.
DOPAC	311 ± 75	24 ± 13	56 ± 17	22 ± 4	11 ± 2	37 ± 7	5 ± 2
3-MT	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
HVA	226 ± 106	18 ± 11	42 ± 6	26 ± 12	n.d.	37 ± 10	1 ± 1
NE	3026 ± 631	180 ± 39	361 ± 114	118 ± 16	40 ± 33	436 ± 114	104 ± 41
E	n.d.	n.d.	16 ± 3	1 ± 2	4 ± 10	n.d.	n.d.
MHPG	1365 ± 745	174 ± 29	718 ± 412	458 ± 299	49 ± 22*	1474 ± 641	—
5-HT	2363 ± 451	90 ± 10	280 ± 78	87 ± 23	39 ± 18	46 ± 23	139 ± 63
5-HIAA	987 ± 243	n.d.	n.d.	1 ± 2	1 ± 2	26 ± 5	16 ± 14

n.d. = not detectable, * MPHG in eWAT has one outlier at 163 ($p < 0.05$).

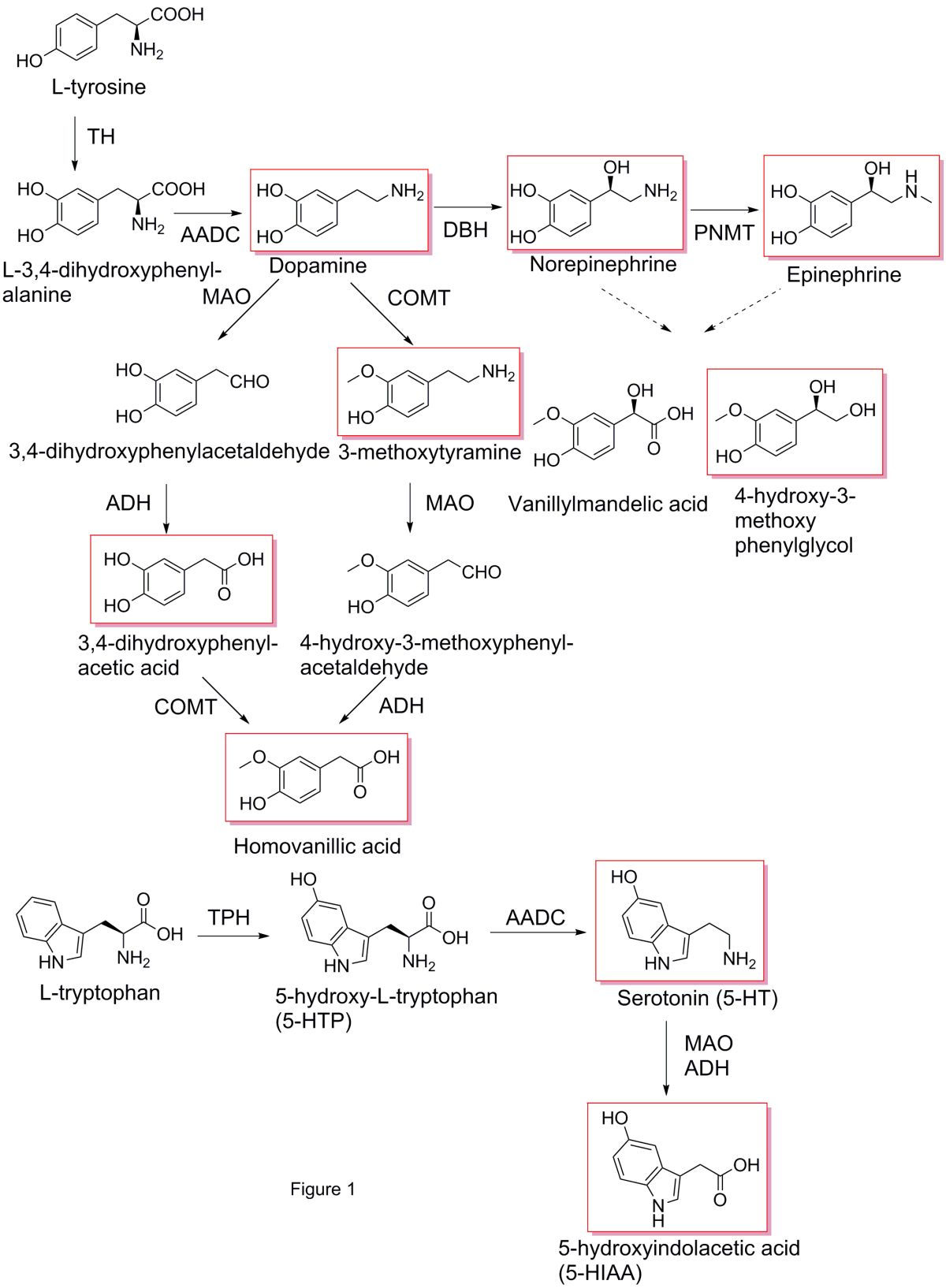

Figure 1

Figure 1. Synthesis and metabolism of MAs. TH: tyrosine hydroxylase, AADC: aromatic L-aminoacid decarboxylase, DBH: dopamine β-hydroxylase, PNMT: phenylethanolamine N-methyltransferase, COMT: catechol-O-methyltransferase, MAO: monoamine oxidase, ADH: aldehyde dehydrogenase, TPH: tryptophan hydroxylase; boxes indicate compounds analyzed with this method.

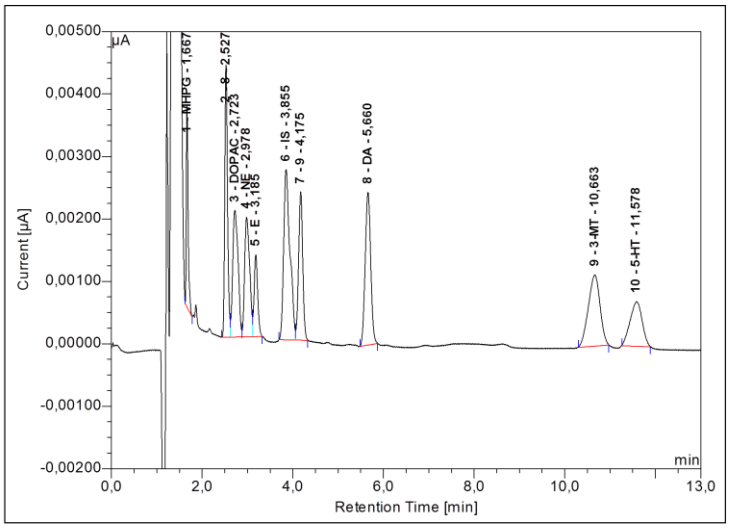

Figure 2

Figure 2. Sample chromatogram of standard solution (1ng 20 µl-1). Peaks in order: MHPG, DOPAC, NE, E, 5-HIAA, IS, HVA, DA, 3-MT, 5-HT.

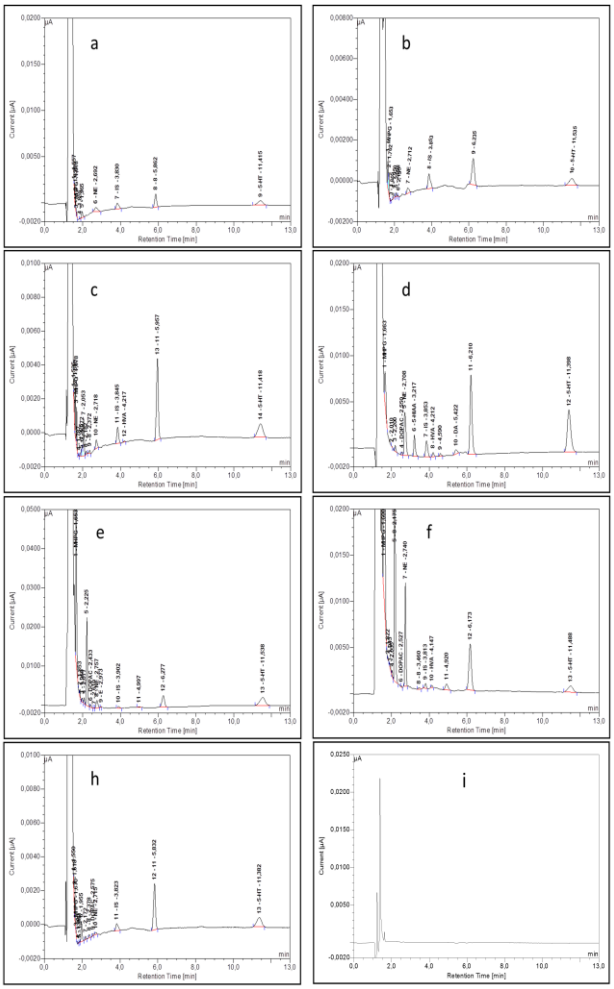

Figure 3: Chromatograms of biological samples. a: soleus; b: EDL; c: gastrocnemius; d: hypothalamus; e: liver; f: pancreas; g: eWAT; i: Blank