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Liquid Chromatography-Tandem Mass Spectrometry in Studies of Neurotransmitters and Their Metabolites in the Brain

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ACADEMIC DISSERTATION

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ABSTRACT

Neurotransmitters are low-molecular weight compounds that chemically transfer the electrical impulse from one neuron to another and are responsible for the normal functioning of the brain. Disturbed balance of neurotransmitters is observed in many neurological disorders, such as Parkinson's and Alzheimer's diseases, and therefore the measurement of neurotransmitters in the brain is needed to understand how these diseases develop and how they can be treated. Neurotransmitters can be extracted from the brains of freely moving, alert animals by microdialysis technique. However, the concentration of neurotransmitters and their metabolites in brain microdialysates is low, and therefore highly sensitive and specific analytical methods are needed. The aim of this study was to develop such liquid chromatography-electrospray ionization-tandem mass spectrometric (LC-ESI-MS/MS) methods for the analysis of rat or mouse brain microdialysates.

The perfusion fluid used in the microdialysis contains a high concentration of inorganic salts (150 mM), whereas the concentration of polar neurotransmitters is low, making demands on chromatographic separation. The inorganic salts cause a suppression effect in ESI, and therefore they must be separated from the analytes to achieve maximal sensitivity of the MS detection. Compounds having the same mass and similar structures as the analytes studied are coextracted from the brain, and these must be separated from each other to ensure specific analysis. In the present study the analytical methods were developed for different chemical classes of neurotransmitters, such as amino acids, acetylcholine (Ach) and biogenic amines and their metabolites.

γ-Aminobutyric acid (GABA) is the predominant inhibitory neurotransmitter in the brain and its concentration in brain microdialysates is low (nM level). However, ESI sensitivity is not optimal for small and polar compounds, such as GABA, and in addition the separation of amino acids from the suppressive salts of the perfusion fluid is problematic. In this study, amino acids were derivatized to enhance their ionization and retention in reversed-phase chromatography. Three different commonly used derivatization reagents (propyl chloroformate (PrCF), butanol and 9-fluorenylmethyl chloroformate (FMOC)) were compared in the analysis of amino acids by LC-MS/MS. The limits of detection (LODs) for all amino acid derivatives were 2–60 times lower than for native underivatized amino acids. The adequate separation of amino acids from the inorganic salts of the perfusion fluid was achieved only after PrCF- and FMOC- but not after butanol derivatization. Of the derivatization reagents tested, PrCF was best suited for the analysis of amino acids by LC-MS/MS, and PrCF-derivatized GABA and nine other amino acids could be easily quantified in rat brain microdialysates. This was the first time that GABA was analysed in rat brain microdialysates by specific LC-MS/MS.

The concentration of Ach in brain microdialysates is low (a few nM). Therefore, in some studies acetylcholinesterase inhibitors have been used to prevent the metabolism of Ach in the brain, and thereby the concentration of Ach in the microdialysate is increased. However, the use of inhibitors is not desirable, since they affect neuronal activity and

distort the natural chemical balance in the brain. In this study, hydrophilic interaction liquid chromatography (HILIC) was utilized in the analysis of Ach and choline (Ch). Ach and Ch showed good retention in the polar diol stationary phase used in HILIC and they were eluted at high (80%) concentration of acetonitrile (ACN) in the eluent, which is favourable to ESI and enabled sensitive analysis of Ach. The limit of quantification (LOQ) for Ach was 0.1 nM, which allowed the analysis of Ach in rat and mouse brain microdialysates without the use of acetylcholinesterase inhibitors.

Neurotransmitters and their phase I metabolites are also known to undergo phase II metabolism in the brain. However, glucuronide and sulfate conjugates have not been studied in brain microdialysates and only nonspecific indirect analytical methods employing acid or enzyme hydrolysis have been used in the analysis of cerebrospinal fluid (CSF) and brain samples. Here sulfate and glucuronide conjugates of dopamine (DA), serotonin (5-HT), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (5-HIAA) were synthesized enzymatically or chemically for reference compounds. LC-MS/MS methods for the analysis of intact sulfate and glucuronide conjugates in rat and mouse brain microdialysates were developed. The methods enabled detection of the different regioisomers of the conjugates, which has not been possible with indirect methods. In the present study, the glucuronidation of the neurotransmitters 5-HT and DA in the brain was shown for the first time. The neurotransmitters were glucuronidated while their acidic phase I metabolites were sulfated.

LIST OF ORIGINAL PUBLICATIONS

This doctoral thesis is based on the following four publications, which will be referred to in the text by their Roman numerals.

I Päivi Uutela, Raimo A. Ketola, Petteri Piepponen and Risto Kostiainen; Comparison of different amino acid derivatives and analysis of rat brain microdialysates by liquid chromatography tandem mass spectrometry. *Anal. Chim. Acta* 633 (2009) 223-231.

II Päivi Uutela, Ruut Reinilä, Petteri Piepponen, Raimo A. Ketola and Risto Kostiainen; Analysis of acetylcholine and choline in microdialysis samples by liquid chromatography/tandem mass spectrometry. *Rapid Commun. Mass Spec.* 19 (2005) 2950-2956.

III Päivi Uutela, Laura Karhu, Petteri Piepponen, Mikko Käenmäki, Raimo A. Ketola and Risto Kostiainen; Discovery of dopamine glucuronide in rat and mouse brain microdialysis samples using liquid chromatography tandem mass spectrometry. *Anal. Chem.* 81 (2009) 427-434.

IV Päivi Uutela, Ruut Reinilä, Kirsi Harju, Petteri Piepponen, Raimo A. Ketola and Risto Kostiainen; Analysis of intact glucuronides and sulfates of serotonin, dopamine, and their phase I metabolites in rat brain microdialysates by liquid chromatography-tandem mass spectrometry. *Anal. Chem.* 81 (2009) 8417-8425.

ABBREVIATIONS

5-HIAA	5-hydroxyindoleacetic acid
5-HT	5-hydroxytryptamine
A	adrenaline
ABA	aminobutyric acid
Ach	acetylcholine
ACN	acetonitrile
ADHD	attention deficit hyperactivity disorder
β-Ala	β-alanine
APCI	atmospheric-pressure chemical ionization
APSI	atmospheric-pressure spray ionization
Arg	arginine
Asn	asparagine
Asp	aspartic acid
CE	capillary electrophoresis
Ch	choline
CNS	central nervous system
CSF	cerebrospinal fluid
COMT	catechol-*O*-methyltransferase
Da	dalton
DA	dopamine
DHPG	3,4-dihydroxyphenylglycol
DOPAC	dihydroxyphenylacetic acid
EC	electrochemical
ESI	electrospray ionization
EtCF	ethyl chloroformate
FL	fluorescence
FMOC	9-fluorenylmethyl chloroformate
G	glucuronide
GABA	γ-aminobutyric acid
GC	gas chromatography
Glu	glutamic acid
Gly	glycine
HFBA	heptafluorobutyric acid
HILIC	hydrophilic interaction liquid chromatography
HMBC	heteronuclear multiple bond correlation
HVA	homovanillic acid
Iso-Ach	(3-carboxypropyl)-trimethylammonium
ISTD	internal standard
LC	liquid chromatography
L-DOPA	L-3,4-dihydroxyphenylalanine
LIF	laser-induced fluorescence
LOD	limit of detection

LOQ	limit of quantification
MAO	monoamine oxidase
MD	microdialysate
MeCF	methyl chloroformate
MeOH	methanol
MHPG	3-methoxy-4-hydroxyphenylglycol
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MTBSTFA	N-(tert-butyldimethylsilyl)-N-methyltrifluoro-acetamide
m/z	mass-to-charge ratio
NA	noradrenaline
NBD-F	7-fluoro-4-nitrobenzoxadiazole
NCI	negative chemical ionization
NDA	naphthalene-2,3-dicarboxaldehyde
NH_4Ac	ammonium acetate
NM	normetadrenaline
NMR	nuclear magnetic resonance
OPA	o-phthalaldehyde
PAPS	3-phosphoadenosine-5-phosphosulfate
PCI	positive chemical ionization
PFP	pentafluorophenylpropyl
PGC	porous graphitic carbon
PrCF	propyl chloroformate
%RSD	relative standard deviation
Rt	retention time
S	sulfate
SCX	strong cation exchange
SIM	selected ion monitoring
S/N	signal-to-noise ratio
SPE	solid-phase extraction
SULT	sulfotransferase
SRM	selected reaction monitoring
Tyr	tyrosine
UDPGA	uridine-5’-diphosphoglucuronic acid
UGT	uridine diphosphoglucuronosyltransferases
UPLC®	Waters ultraperformance liquid chromatograph
UV	ultraviolet

1. INTRODUCTION

There are about 100 billion nerve cells in the human brain and over 99% of the communication between neurons is based on chemicals called neurotransmitters [1]. Neurons vary in shape and size, but basically a neuron has a cell body with two types of projections: dendrites bringing information to the cell body and an axon carrying the information from the cell body. When electrical impulses arrive at the nerve ending, neurotransmitters are released from the presynaptic neuron to a synaptic cleft, a microenvironment between neurons, and they bind to specific receptors of the postsynaptic neuron. The binding either activates, producing an electrical impulse, or deactivates, hindering the formation of electrical impulses in the postsynaptic neuron. In other words, the electrical impulse from one neuron to another is chemically signalled by neurotransmitters. To terminate the signal, neurotransmitters are enzymatically inactivated or they are transferred from the synaptic cleft by active uptake mechanisms. The neurotransmitters taken back to the presynaptic neuron are either metabolized or restored for a new neurotransmission process. Molecules with different structures function as neurotransmitters, and they are divided into different chemical classes such as biogenic amines, peptides and amino acids. Neurotransmitters are responsible for the functioning of the central nervous system (CNS); mood, thought, learning, and memory, for example. Disturbed balance of neurotransmitters in the brain has been observed in various neurological disorders, such as Parkinson's disease [2], Alzheimer's disease [3], and schizophrenia [4]. Understanding of brain functioning in normal and diseased states is essential to the development of treatment strategies, and knowledge of where neurotransmitters are released and at what rate is desired. However, sampling from the brain is challenging. A microdialysis technique has been used for the extraction of neurotransmitters from freely moving and alert animals. The concentration of neurotransmitters in brain microdialysates (MDs) is then measured, either on-line or off-line. However, the concentrations are low and thus highly sensitive and specific analytical methods are needed in the area of brain research.

1.1 Neurotransmission

Neurotransmitters can be divided according to their chemical structure into catecholamines, amino acids, indoleamines, neuropeptides and acetylcholine (Ach). The chemical signalling differs among neurotransmitters, and the speed of synaptic signal transmission is either fast (below 1 ms) or slow (from hundreds of milliseconds to minutes) [1]. The rapid transmission operates through ligand-operated ion channel receptors (ionotropic channels) that allow the flow of chloride, sodium, potassium or calcium ions into the postsynaptic neuron. The inside of the neuron is more negatively charged than the outside. The binding of an excitatory neurotransmitter into the ion channel causes the flow of positive ions into the postsynaptic neuron, which is then depolarized. If the depolarization is high enough, an action potential is formed and a rapid

electrical impulse travels down the neuron. Half of all the fast synapses in the brain are excitatory and the main neurotransmitter is the amino acid glutamic acid (Glu). Another amino acid, γ-aminobutyric acid (GABA), is mainly responsible for fast inhibitory transmission and the binding of GABA opens an ion-channel, which allows the chloride ions to flow inside the cell. The neuron is hyperpolarized and the formation of electrical pulses in the postsynaptic neuron is hindered. Synaptic transmission is slow in the majority of neurotransmitters, including GABA and Glu, when they bind to the G-protein-coupled receptor (metabotropic receptor). Binding activates secondary messengers, which in turn activate distinct classes of protein kinases. The consequence of these complex cascades is the control of production of fast- and slow-acting receptors and ion pumps that restore the ionic equilibrium after hyperpolarization or depolarization. Slow synaptic transmission also affects transcription factors in the cell nucleus, resulting in long-term changes in the nerve cell that are likely needed in learning and memory processes.

Signalling between neurons does not always occur in a way that neurotransmitters are released from the axon terminals of the presynaptic neuron and alter the signalling of the postsynaptic neuron. Compounds, such as nitric oxide and lipid derivatives (anandamide, 2-arachnoidyl glycerol), have been identified in the brain where they act as retrograde messengers that are released from the postsynaptic neuron and control presynaptic activity [5]. The “common neurotransmitters” can also be released nonsynaptically from localized axonal swellings and diffuse to the site of activity in the extracellular space. Neuropeptides, for one, are released almost from the entire surface of the neuron and they diffuse to the site of activity through the extracellular space. Neuropeptides are from 3 to 100 amino acid residues long, and their signalling specificity is based on high binding affinity for their receptors. The neural activity aroused by neurotransmitters can also be modified by other compounds such as neuroactive steroids [6, 7]. Neuroactive steroids may be synthesized in the brain or periphery, and among their many targets are the amino acid receptors, but they bind to different sites than the actual neurotransmitters. The modulatory effects of different neurosteroids can be inhibitory or excitatory.

1.1.1 Synthesis and phase I metabolism of neurotransmitters

In the early 20th century, there was ongoing debate on whether communication between neurons was chemical or electrical in nature and Ach was the first neurotransmitter identified in the CNS [8]. The synthesis of Ach from choline (Ch) and acetyl-coenzyme A is catalysed by choline acetyltransferase in the presynaptic neuron [9, 10]. Ach is stored in vesicles, which are fused to the presynaptic membrane of the neuron after the electrical impulse, and Ach is released to the synaptic cleft. The activity of Ach is quickly terminated by acetylcholinesterase present in membranes of the pre- and postsynaptic neurons, and Ach is hydrolysed to Ch and acetate (Fig. 1). Ch is actively taken back to the presynaptic neuron for the synthesis of new Ach. Ach is known to be involved in learning, memory, temperature and blood pressure regulation. Several diseases are also related to

malfunctioning of the cholinergic system, such as Huntington's, Alzheimer's and Parkinson's diseases.

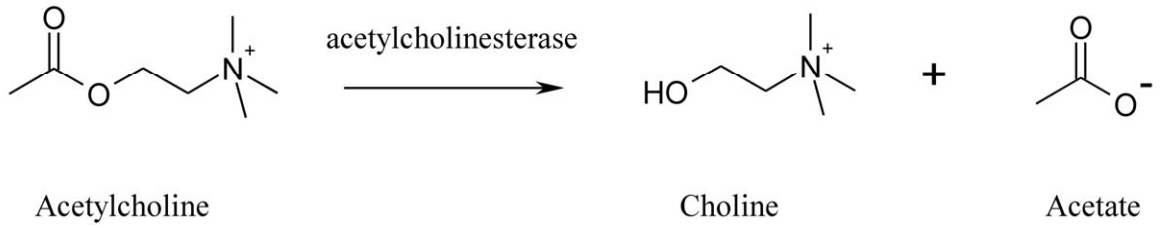

Figure 1. *Metabolism of acetylcholine to choline and acetate by acetylcholinesterase.*

Amino acids are an important and abundant class of neurotransmitters in the brain [11, 12]. However, they were identified as neurotransmitters much later than Ach and the biogenic amines. The most important amino acid neurotransmitters are excitatory Glu and aspartic acid (Asp), and inhibitory GABA and glycine (Gly) (Fig. 2). The metabolism of excitatory and inhibitory amino acids is closely related. GABA is formed from Glu by an enzyme called glutamate decarboxylase. GABA and Glu for one can be metabolized to aspartate. The action of GABA and Glu in the synaptic cleft is terminated by uptake to the presynaptic neuron or glial cells, which provide support and nutrition for neurons. Glu is neurotoxic, which may be why Glu in glial cells is converted to nontoxic glutamine, which in turn can be synthesized back to Glu in the presynaptic neurons. In addition to acting as a neurotransmitter, Glu is associated with metabolic regulation in the brain, which complicates study of the neurotransmitter role of Glu. Glu is associated with learning and memory functions of the brain as well as neurological disorders such as cerebral ischaemia, hypoxia and epilepsy [12]. Malfunctioning of GABA excretion is observed in the pathophysiology of epilepsy and anxiety, and altered concentrations of Gly, Glu, and GABA are observed in schizophrenia.

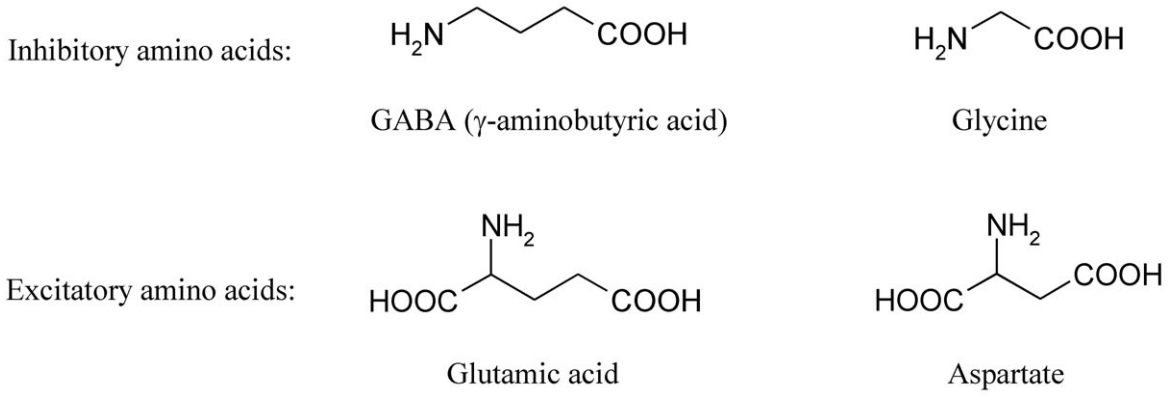

Figure 2. *Structure of main inhibitory and excitatory amino acids in central nervous system.*

Catecholamines, such as dopamine (DA), noradrenaline (NA) and adrenaline (A), are synthesized from tyrosine (Tyr) [13]. The rate limiting step is hydroxylation of Tyr to L-3,4-dihydroxyphenylalanine (L-DOPA) by tyrosine hydroxylase. The formation of DA from L-DOPA is catalysed by the aromatic L-amino acid decarboxylase. DA itself acts as a neurotransmitter, but it is also a precursor for synthesis of NA (Fig. 3) [13]. The formation of serotonin (5-hydroxytryptamine, 5-HT) begins with hydroxylation of tryptophan by tryptophan hydroxylase and is continued by the aromatic L-amino acid decarboxylase [14]. The activity of 5-HT and DA in the synaptic cleft is terminated mainly by active uptake mechanisms in the presynaptic neurons [15]. Part of the DA and 5-HT is also metabolized through monoamine oxidase (MAO) and aldehyde dehydrogenase catalysed reactions to dihydroxyphenylacetic acid (DOPAC) and 5-hydroxyindoleacetic acid (5-HIAA), respectively (Fig. 3). DOPAC can be further metabolized to homovanillic acid (HVA) by catechol-*O*-methyltransferase (COMT). The major metabolite of NA in the CNS is 3-methoxy-4-hydroxyphenylglycol (MHPG). 5-HT-excreting neurons are found in almost all brain areas and therefore 5-HT is associated with regulation of emotional, feeding and cognitive behaviour, sleep, thermoregulation etc. as well as pathological states such as depression, mania, migraine and schizophrenia [14]. Catecholamines, especially DA, are involved in attention deficit hyperactivity disorder (ADHD), schizophrenia and Parkinson's disease [16]. It is also known that DA plays a central role in the mechanism of action of drugs of abuse.

1.1.2 Sulfation and glucuronidation of neurotransmitters

Enzyme-catalysed metabolism is commonly divided into phase I and phase II reactions. A small polar functional group is added or exposed in phase I reactions, which include oxidation, reduction and hydrolysis. Phase II reactions are called conjugation reactions, in which a polar moiety is attached to the molecule. Glucuronidation and sulfonation (more commonly called sulfation) are common phase II metabolism reactions (Fig. 4). Glucuronidation is catalysed by uridine diphosphoglucuronosyltransferases (UGTs), which are membrane-bound enzymes of the endoplastic reticulum [17]. In the reaction the glucuronic acid moiety is transferred from the uridine-5'-diphosphoglucuronic acid (UDPGA) to a wide variety of compounds that contain hydroxyl, carboxyl, amine or thiol groups. UGTs exhibit tissue-specific expression but are mainly expressed in the liver. Isoforms of UGTs 1A6, 2A1 and 2B7 have been found in human [18] and UGT1A6 in rat brain [19]. UGTs 1A6 and 2B7 are known to catalyse the glucuronidation of 5-HT, the former having a much higher glucuronidation rate than the latter enzyme [20]. So far glucuronide conjugates of neurosteroids have been detected in mouse brain [21] and dopamine glucuronide (DA-G) in rat [22] and human cerebrospinal fluid (CSF) [23].

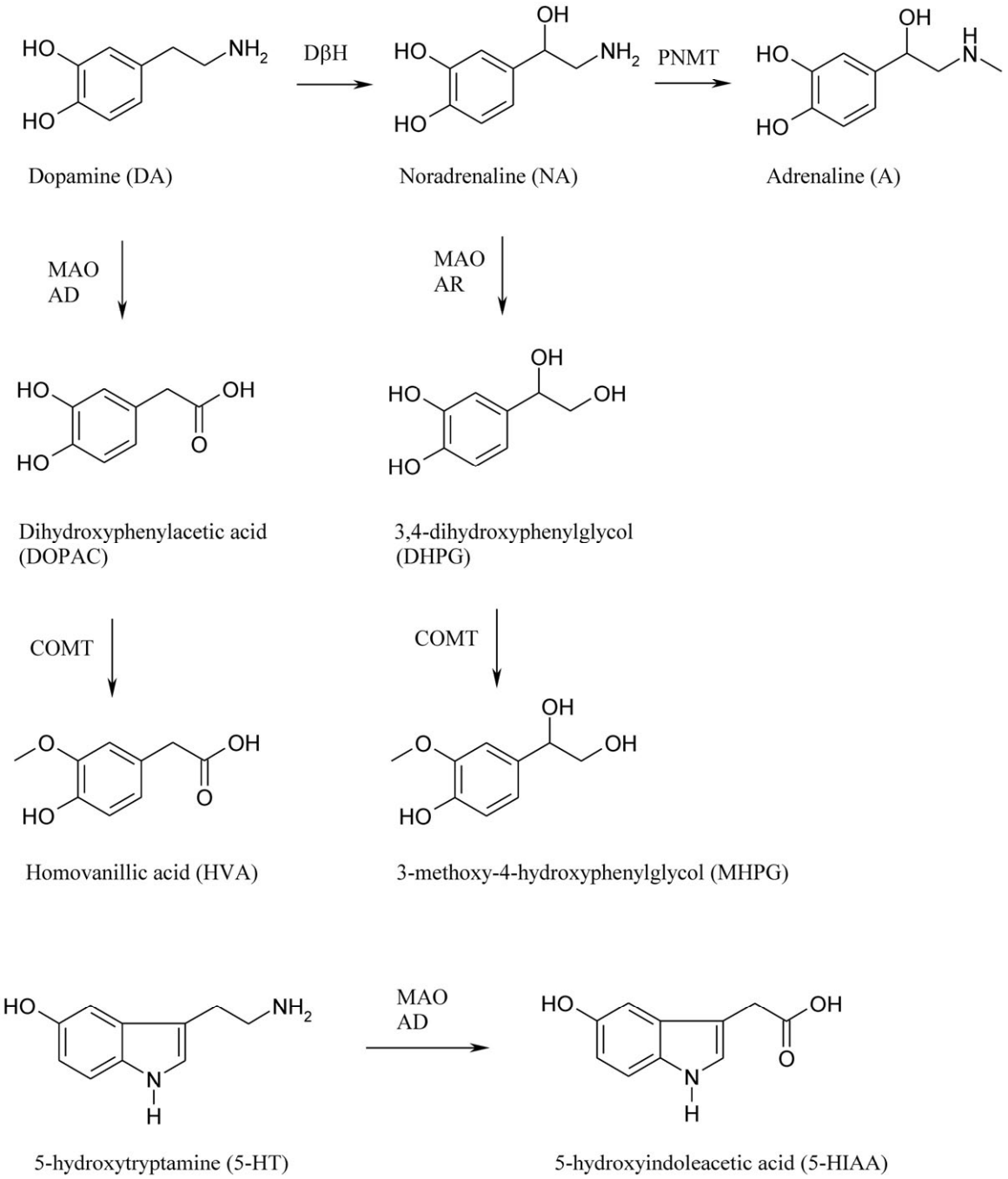

Figure 3. *Structures of biogenic amine neurotransmitters and their metabolites in the central nervous system.*

Sulfation is catalysed by cytosolic sulfotransferase (SULT) enzymes, which are divided according to their amino acid sequence and enzyme function into the phenol (SULT1) and steroid (SULT2) sulfotransferase families [24]. Most commonly the hydroxyl group of the substrate is sulfated and the cosubstrate needed in the reaction is 3'-phosphoadenosine-5'-phosphosulfate (PAPS). Sulfation of phenols is catalysed by the SULT1 family, and SULTs 1A1, 1A2 and 1A3 have been found in the human brain. The isoform SULT1A3, existing only in humans and other very close primate relatives, has high selectivity and catalytic efficiency for DA. In rat brain the activity of phenol-sulfating SULT is also observed [25] and sulfate conjugates of DA [26] and monoamine metabolites, such as DOPAC-sulfate (DOPAC-S), HVA-S, MHPG-S and 5-HIAA-S have been detected [27-29]. Usually metabolism leads to inactivation, but some neurosteroid sulfates are known to act as neuromodulators in the brain [30]. The sulfated form may also be exploited in the synthesis of other neurotransmitters as reported for DA-S, which is a substrate of NA-forming dopamine-β-hydroxylase [31].

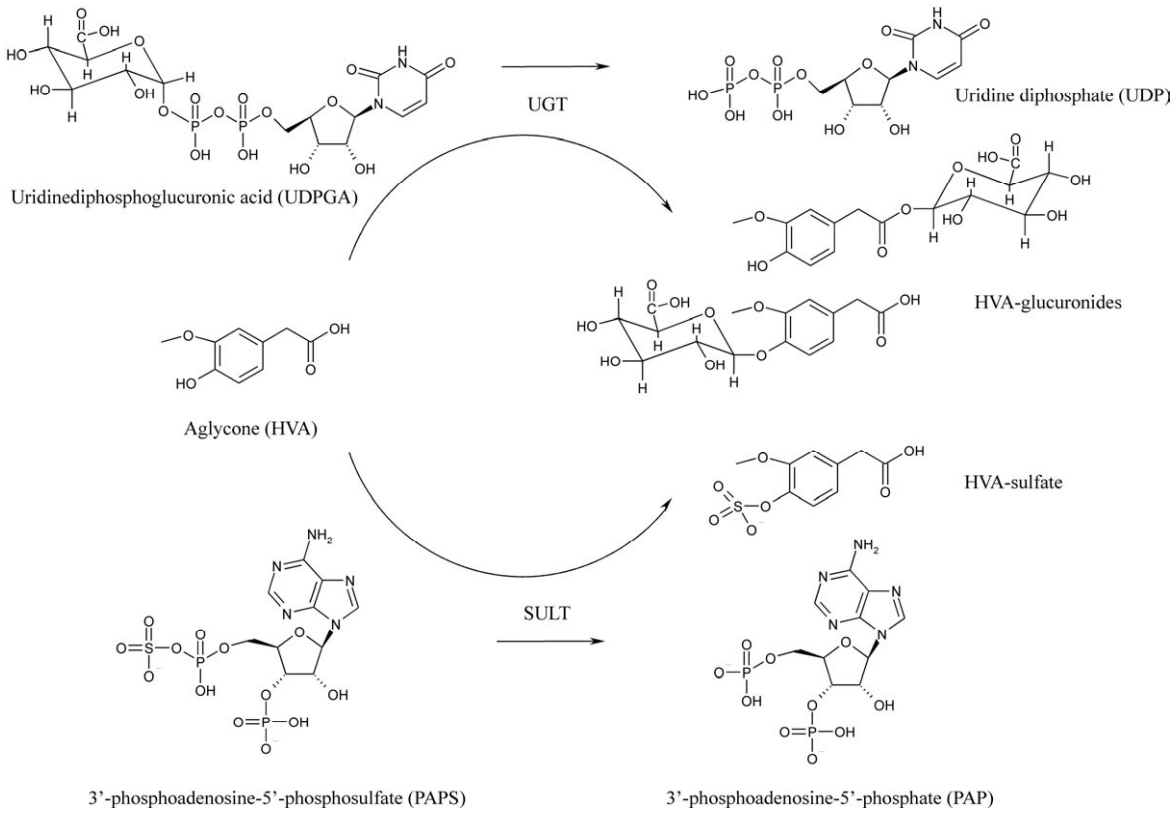

Figure 4. *Enzyme-catalysed glucuronidation and sulfation of HVA.*

1.2 Techniques for studying neurotransmission

Monitoring of chemical changes in the brain is challenging. The aim is to measure the concentration of neurotransmitters and their metabolites in local brain areas with high sensitivity and specificity. The measurement of changes in concentrations at short time intervals is also desired [32]. One way is to analyse neurotransmitters in the brain tissue. Typically, a brain sample is homogenized in acid and water-soluble neurotransmitters are extracted to the liquid phase [12]. The release and changes in neurotransmitter concentrations cannot be measured this way and only a snapshot can be achieved [5]. The proportion of extracellular and intracellular neurotransmitters likewise cannot be identified, and postmortem changes in the concentrations of neurotransmitters are possible. Samples taken from the CSF better reflect the release of neurotransmitters, but the concentrations of neurotransmitters provide only a rough temporal estimation of the release throughout the entire CNS. Microdialysis and voltammetry provide more localized information on brain neurotransmission, and these techniques are mainly used in neurochemical monitoring.

In voltammetry a carbon electrode (3 µm in diameter) is inserted into a region of interest in the animal brain [33]. Voltammetry is based on oxidation and reduction reactions of electroactive compounds of the brain at the surface of the electrode. Oxidation at a specific potential generates a current, whose magnitude is used in the estimation of the concentration. However, the differences in oxidation potentials of compounds found in the brain are small, complicating the specific analysis. Currently, fast cyclic voltammetry is used, in which the potential of the electrode is linearly ramped and compounds having slightly different oxidation potentials can be separated. More specificity can also be obtained using enzyme-based sensors, in which the enzyme specifically binds the analyte studied and the end product of the enzymatic reaction is oxidized at the surface of the electrode. The greatest advantage of the voltammetry technique is the high temporal resolution of 100 ms or less [34].

Microdialysis has been widely used in the sampling of neurotransmitters and their metabolites in defined brain areas [32, 35, 36]. Even though the temporal resolution is lower than in voltammetry, the identity and concentration of neuroactive compounds can be more accurately determined by selecting specific analytical techniques for the measurement of brain microdialysates. In microdialysis a guide cannula is surgically implanted into a region of interest in an animal's brain. After the animal has recovered from the surgery, a semipermeable dialysis membrane is inserted into the guide cannula [37]. The sampling is performed on a freely moving, unanaesthetized animal by pumping physiological perfusion fluid at a low flow rate (0.5–2.5 µl/min) through the dialysis membrane [32, 35, 36]. The low-molecular weight and water-soluble compounds in the extracellular fluid of the brain are extracted to the perfusion fluid by passive diffusion according to the concentration gradient. The extracellular fluid of the brain contains synaptically released neurotransmitters and their metabolites, as well as compounds from

nonsynaptic sources [36]. The molecular mass cutoff of membranes varies from 5 to 100 kilodaltons (kDa), preventing the entering of proteins into the dialysate. The recovery of compounds is dependent on many variables, such as the temperature, molecular weight and charge, flow rate of the perfusion fluid, surface area and material of the dialysis membrane etc. [35]. The concentration of neurotransmitters in the extracellular fluid can be estimated, but the absolute value for *in vivo* recovery of compounds in microdialysis is difficult to determine. Another disadvantage of the microdialysis technique is that the invasive implantation of the microdialysis probe may evoke inflammation [32]. The concentration of compounds in microdialysates is also lower than in the extracellular fluid, due to the diluting effects of dialysis, making demands on the analytical techniques used in the analysis.

1.3 Analysis of neurotransmitters in brain microdialysates

The concentration of neurotransmitters in microdialysates is low (in the fmol range) and the sample volume is limited (typically < 40 µl) [38, 39]. The perfusion fluid used in microdialysis contains a high concentration (150 mM) of inorganic salts, which must be separated from polar neurotransmitters to ensure reliable detection and quantification of the analytes. Separation of the analytes studied from other endogenous compounds coextracted from the brain is also needed. Usually neurotransmitters have been analysed by liquid chromatography (LC) connected to electrochemical (EC), fluorescence (FL), ultraviolet (UV) or electrospray ionization-mass spectrometric (ESI-MS) detectors. The retention of neurotransmitters and their metabolites in reversed-phase chromatography is poor and therefore ion-pair chromatography, cation-exchange chromatography, and hydrophilic interaction liquid chromatography (HILIC) employing polar stationary phases have been used to achieve adequate retention and separation [38, 39]. The separation of polar compounds in HILIC is believed to be based on different partitioning of the analyte between the hydrophobic eluent and a water-rich liquid layer formed on the polar stationary phase. Detection of lower concentrations of neurotransmitters in microdialysates has been achieved by downscaling the column diameter [40]. However, only the MS of the detectors used provides sufficient specificity in the analysis of neurotransmitters, since the analyte is identified by both retention time (rt) and molecular weight. Using tandem mass spectrometry (MS/MS), the specific dissociation pathway of the molecule is followed, which further increases the specificity, and the limit of detection (LOD) is also improved compared with MS detection. Volatile eluents must be used in the LC-MS/MS analysis and therefore the nonvolatile concentrated buffers used in ion-exchange chromatography and nonvolatile ion-pair reagents are not an option. The inorganic salts of the perfusion fluid cause ion suppression in the analysis of brain microdialysates by LC-ESI-MS/MS and they must be separated from the analytes. Other endogenic compounds extracted from the brain may also coelute with the analytes studied and alter the ESI process. Therefore possible suppression effects must be studied individually for each analyte to ensure reliable quantification. The analytical challenges

related to measurements of amino acids, Ach and monoamine neurotransmitters in microdialysates are described here in more detail.

1.3.1 Amino acids

The concentration of amino acids in microdialysates is much higher (from nM to µM) than other neurotransmitters, such as Ach, 5-HT and DA (low nM). There have been speculations on whether the concentrations of GABA and Glu, the most often analysed amino acids in microdialysates, reflect the neuronal release of these neurotransmitters or do the amino acids enter into the extracellular fluid also from non-neuronal sources [41-43]. Two criteria should be fulfilled, provided the neurotransmitter in the microdialysate has originated from neuronal release. The concentration of neurotransmitter in the microdialysate should be decreased by 70–90%, when the formation of an action potential in the nerve cell is blocked by the Na^+-channel blocker tetrodotoxin or the action of Ca^{2+} is blocked by the use of the Ca^{2+} antagonist Mg^{2+} or Ca^{2+}-chelating ethylenediaminetetraacetic acid. Ca^{2+} ions under normal conditions flow into the presynaptic neuron during the action potential and are needed for the release of neurotransmitters into the synaptic cleft. The concentration of DA, 5-HT and Ach is clearly decreased after a 10–15 min infusion of tetrodotoxin and these neurotransmitters also show a clear response to Ca^{2+} depletion [42, 43]. However, a statistical difference in the concentration level of GABA is seen only 50–80 min after the tetrodotoxin dosage, indicating that GABA is at least partly originated from non-neuronal sources [41-43].

Amino acids have been analysed by LC, capillary electrophoresis (CE) and GC [12]. Derivatization of nonvolatile amino acids and sample cleaning is required in GC analysis. Derivatization is also often employed in the analysis of amino acids by LC and CE to enhance the sensitivity of detection. The most commonly used derivatization reagent is o-phthalaldehyde (OPA), and amino acid derivatives can be detected by FL [44, 45] or EC [46, 47]. However, some of the amino acid derivatives formed with OPA are labile [48] and therefore naphthalene-2,3-dicarboxaldehyde (NDA), which forms more stabile derivatives, has been used in the analysis by CE-laser-induced fluorescence (LIF) [49] and LC-FL [50, 51]. Good sensitivity is required from the analytical method so that smaller sample volumes can be analysed and thereby the rapid changes in concentration of amino acids in the brain can be monitored. In the majority of studies using LC-EC [47, 52, 53] or LC-FL [50, 54] the samples are collected at 5–30-min time intervals (sample volume 2–30 µl), but a significantly higher sampling rate (10 s, 200 nl sample volume) was reported, using capillary LC-amperometry [46]. The fast analysis times achieved with CE (< 20 s) has enabled the on-line analysis of amino acids in brain microdialysates [55-57].

The concentrations of GABA reported in the literature have varied greatly (9–170 nM) in rat brain striatum microdialysates analysed by LC-EC [47, 52], LC-FL [58] or CE-LIF [49, 55, 56, 59]. Rea *et al.* [58] suggested that one reason for the high variation in GABA concentration is the difficulty in separating GABA from the endogenous impurities

present in microdialysates with nonspecific analysis methods. Higher specificity can be achieved using MS detection but in the study of Ma *et al.* [60] the LOD (10 µM, 200 pmol) was too high for the analysis of GABA in rat and mouse brain microdialysates. A lower LOD (48 nM, 1 pmol) for GABA by LC-MS/MS was achieved after precolumn derivatization with 7-fluoro-4-nitrobenzoxadiazole (NBD-F) [61]. The method was applied for the analysis of human plasma and CSF samples in which the concentration of GABA was much higher (> 400 nM) than in rat brain microdialysates. A capillary-HILIC-MS/MS method was used for the quantitative analysis of underivatized GABA sampled from monkey brain [62]. Even though the LOD for GABA in the capillary-HILIC-MS/MS method was 4 nM (1.2 fmol), the quantitative analysis of rat brain microdialysates might be problematic, because five-fold dilution of the microdialysis sample with ACN was needed to ensure good chromatographic performance and thereby the lowest detectable concentration of GABA in brain microdialysate is 20 nM. Isomers of GABA, α-and β-ABA, are also coextracted from the brain, and the separation of these compounds from GABA must be taken into account, even when using specific LC-MS/MS [46, 55].

1.3.2 Acetylcholine

Ach is quickly metabolized to Ch and acetate by acetylcholinesterase after release in the synaptic cleft and hence the concentration of Ach in the microdialysates is low (0.1–6 nM) [63, 64]. Therefore in some studies acetylcholinesterase inhibitors have been used to increase the concentration of Ach in the extracellular fluid of the brain [65]. However, the inhibitors may affect neuronal activity and alter the effects of different drugs on Ach release. To avoid the use of acetylcholinesterase inhibitors, sensitive analytical methods for the measurement of Ach have been developed. The concentration of Ch in the extracellular fluid is high (0.6–5 µM) [66, 67] and chromatographic separation from Ach is needed, especially with nonspecific detectors.

Gas chromatography (GC) has been used for the analysis of Ach in brain samples [9], but the analysis of Ach in brain microdialysates has not been possible without the use of acetylcholinesterase inhibitors in the perfusion fluid, due to the high LOD of the GC-MS method (10-40 nM, 0.5–2 pmol) [68]. The most widely used method for the analysis of Ach in microdialysates is LC-EC, and the LODs are low enough (0.2–2 nM, 2–10 fmol) for the analysis of brain microdialysates without the use of acetylcholinesterase inhibitors [64, 65, 69]. However, better sensitivity and specificity is achieved with LC-ESI-MS/MS, the LOD being 0.075–1 fmol (7.5–100 pM) [63, 70, 71], and by using capillary LC columns attomol levels (8–15 amol, 40–50 pM) can be achieved [62, 72] (Table 1). Furthermore, LC-MS/MS provides direct detection of Ach and Ch, whereas when EC is used, the analytes must be converted into hydrogen peroxide using enzyme-catalysed reactions. Separation of Ach from the inorganic salts of the microdialysis perfusion fluid is necessary to avoid suppression effects in ESI and contamination of the ion source. Even though MS/MS provides specificity, the chromatography plays an essential role in the analysis of Ach, because endogenous compounds having the same mass and similar

collision-induced dissociation as Ach and acetyl-β-choline, are extracted from the brain [63, 70]. Acetyl-β-choline is commonly used as an internal standard (ISTD). The chromatographic separation of these endogenous compounds from Ach and the ISTD is clearly needed to guarantee reliable quantification.

Cation-exchange chromatography [70, 72-74] and HILIC [62, 71] are most commonly used in the LC-MS/MS analysis of Ach in microdialysates. In cation-exchange chromatography concentrated buffers must be used, and they may suppress the ESI process. In HILIC, polar stationary phases and aqueous mobile phases containing more than 50% organic solvents are used [75]. The high concentration of organic solvent facilitates the desolvation and compound ionization in ESI. The use of capillary columns has enabled MS/MS detection at the attomol level with both cation-exchange and HILIC separation methods [62, 72].

Ion-pair chromatography has been used in few studies for the analysis of Ach [63, 76]. However, the ion-pair reagents, having limited volatility, can contaminate the MS ion source and deteriorate the performance of ESI, resulting in low sensitivity and signal drop in a short time [39]. Atmospheric-pressure chemical ionization (APCI) is less prone to signal suppression than ESI. Since the Ach is positively charged, regardless of the pH (due to a quaternary amine group), the APCI source can be used without a discharge current. This is called atmospheric-pressure spray ionization (APSI), and it was exploited in a recent study, where Ach could be routinely analysed in rat brain microdialysates using trifluoroacetic acid in the eluent [77]. The ion pair reagent trifluoroacetic acid causes suppression in ESI, whereas in APSI the signal intensity of Ach was even increased.

1.3.3 Biogenic amines and their metabolites

The concentrations of biogenic amine neurotransmitters and also their phase I metabolites in brain microdialysates are used in determination of the activity of DA-, NA- and 5-HT-excreting neuronal systems. These neurotransmitters and their metabolites are also known to undergo phase II conjugation reactions in the brain: DA-G was detected in rat CSF [22] and sulfates of DA, MHPG, 5-HIAA, DOPAC and HVA have been measured in rat brain samples [26-29]. The sulfate and glucuronide conjugates in brain have mostly been analysed by indirect analysis methods employing acidic [78-84] or enzymatic hydrolysis [26-29]. Even though the type of conjugate can be investigated with greater certainty after enzyme than after acid hydrolysis, the presence of the conjugate is still difficult to confirm by indirect methods, especially if the concentration of the conjugate is low compared with that of free aglycone. Few methods are available for the analysis of intact sulfates or glucuronides of neuronal compounds, probably due to lack of commercial standards: analysis of DA-, NA- and 5-HT-Ss by ion-pair chromatography-EC [85] and LC-UV-FL for the determination of intact 5-HT-G [86]. However, the LODs with these methods may be too high for the analysis of brain microdialysates being 50 nM (1 pmol) for DA-, NA-, and 5-HT-Ss [85].

DA, NA, A and 5-HT have usually been analysed using LC-EC, LC-FL, LC-chemiluminescence or LC-MS [87]. CE-LIF has been used for the analysis of DA and 5-HT in brain microdialysates [88, 89] and an on-line analysis of DA was also presented [90]. The retention of polar catecholamines in reversed-phase chromatography is poor and therefore ion-pair reagents (alkylsulphonic acids) were used in the eluent to improve retention and separation [91]. Cation-exchange chromatography was also used for the analysis of biogenic amines in microdialysates [92]. However, these chromatographic methods are not preferred for sensitive ESI-MS detection, because nonvolatile ion-pair reagents and concentrated buffers are used as eluents. To increase the sensitivity of LC-MS/MS analysis, volatile eluents such as formic acid (HCOOH) and ammonium acetate (NH_4Ac) have been used in reversed-phase chromatography [93-95]. Since formate and acetate form only weak ion-pairs with biogenic amines, the retention of neurotransmitters in reversed-phase chromatography is poorer than with alkylsulphonic acids. Therefore, more polar stationary phase, such as polyhydroxyethyl aspartamide [62], has been used to enhance retention.

Biogenic amine neurotransmitters are easily oxidized, and therefore they have usually been detected by EC. Catecholamines and 5-HT also have native fluorescence, but derivatization has been used to improve separation and ensure sensitive detection by LC-FL (4.2–9.5 pM, 42–96 amol), because the concentrations of these neurotransmitters in brain microdialysates are low (fmol amounts) [96]. Higher selectivity is provided by LC-MS/MS as shown by Hows *et al.* [93], who analysed rat brain microdialysates by both LC-EC and LC-MS/MS. The concentration of DA in microdialysates was 5.5 nM when analysed by LC-EC and only 0.47 nM when analysed by LC-MS/MS, indicating that some endogenous impurities were interfering in the quantitative analysis with LC-EC. The LODs for NA and 5-HT by LC-MS/MS (0.2–1 nM, 2–10 fmol [93]) were too high for quantification of the basal levels of these neurotransmitters in rat brain microdialysates. Similar LODs were achieved by LC-EC [97], but by using capillary LC-EC the LOD for 5-HT could be decreased to 84 pM (84 amol) [98]. In a recent study DA, NA and 5-HT were derivatized by deuterated acetaldehyde and the LODs (30 pM, 600 amol) were as many as 20–100 times lower than those for native neurotransmitters, using ultraperformance LC (UPLC®)-MS/MS [99]. With this UPLC®-MS/MS method the concentrations of DA, NA and 5-HT could be determined in microdialysates collected from the medial prefrontal cortex of rat brain.

Table 1. *Utilization of mass spectrometry in the analysis of neurotransmitters in the brain. The analyses were carried out in SRM mode unless otherwise stated with asterisk (*).*

Amino acids

Analytes	Separation	Column	Eluents	Derivati-zation	Ionization	Inj. [µl]	LOD (nM)	Sample	Ref
GABA, β-ALA	GC*	CP-Sil 88		MeCF	NCI, NH_3		GABA: 5	Human lumbar CSF	[100]
Amino acids	GC*	HP-5ms		EtCF	EI		Glu: 1 pmol	Rat brain	[101]
Amino acids, GABA, Glu, Gly, Ser, Cys	GC*	HP-5ms		MTBSTFA	PCI (NH_3)			Rat brain MD	[102]
D- and L-serine	LC	C-18 (2.1×40 mm, 5 µm)	15 mM NH_4Ac MeOH:ACN	Marfey's reagent	ESI-	50	LLOQ 95	Rat brain MD	[103]
Ach, 5-HT, DA, GABA, Glu, Asp	Cap-LC	HILIC (polyhydroxyethyl aspartamide) (0.2×300 mm)	20 mM NH_4form + 1% HCOOH ACN	-	ESI+	0.3	Ach: 0.05 5-HT: 0.5 DA: 1 GABA: 4 Glu: 20 Asp: 50	Monkey brain Push-pull sampling	[62]
GABA	Cap-LC	C-18 (0.25×50 mm, 5 µm)	0.1% HCOOH ACN	NBD-F	ESI+	20	GABA: 49	Human CSF	[61]
D-L-Glu, -Ser, - Asp	Cap-LC	Chirobiotic TAG (0.25×170 mm)	0.012% TFA MeOH	NBD-F	ESI+	5	Glu: 660	Rat brain	[104]
GABA, Glu	LC* (SIM)	C-18 (4.6×150 mm, 5 µm)	100 mM NH_4Ac ACN	-	APCI+	20	GABA: 10 µM Glu: 14 µM	Rat brain	[60]
GABA, Glu, Arg, Asn, Ser, Gly, Thr, Ala, α- and β-ABA	LC	C-18 (2.1×50 mm, 3 µm)	0.1% HCOOH ACN	PrCF	ESI +	15	0.5 – 2	Rat brain MD	I

NBD-F = 7-fluoro-4-nitrobenzoxadiazole
MTBSTFA = N-(tert-butyldimethylsilyl)-N-methyltrifluoro-acetamide
Marfey's reagent = 1-fluoro-2,4-dinitrophenyl-5-L-alaninamide
MD = microdialysate
NCI = negative chemical ionization
PCI = positive chemical ionization
Chirobiotic TAG = Teicoplanin aglycon CSP
PrCF = propyl chloroformate
MeCF= methyl chloroformate
EtCF = ethyl chloroformate

Acetylcholine

Analytes	Separation	Column	Eluents	Derivati-zation	Ionization	Inj. [µl]	LOD (nM)	Sample	Ref
Ach	GC	Megabore DB-5		Benzene-thiolate	PCI, CH_4	47	Ach: 10-40	Rat brain MD	[68]
Ach	Cap-LC	SCX 0.075×50 mm, 5 µm)	25 mM NH_4Ac+ 25 mM NH_4form, pH 4 MeOH	-	ESI +	5	Ach: 0.04	Rat brain MD	[72]
Ach, Ch, iso-Ach	LC	SCX (2.1×150 mm, 5 µm)	15 mM NH_4Ac+ 10 mM NH_4form, pH 4 ACN	-	ESI +	20	Ach: 0.01 Ch: 0.1	Rat brain MD	[70]
Ach	LC	C-18 (2.1×100 mm, 3.5 µm)	0.05% TFA ACN	-	APSI+	20	Ach: 0.15 LOQ	Rat brain MD	[77]
Ach	LC	DuetC18/SCX (2.1×150 mm, 5 µm)	25 mM NH_4Ac+ 25 mM NH_4form, pH 4 ACN	-	ESI+	10	Ach: 0.1	Rat brain MD	[74]
Ach	LC	C-18, ion pair (2.1×50 mm, 5 µm)	20 mM NH_4form, pH 3 0.1% HFBA ACN	-	ESI+	50	Ach: 0.3	Rat brain MD	[76]
Ach, Ch	LC	HILIC, diol (4×125 mm, 5µm)	20 mM NH_4form, pH 3.3 ACN	-	ESI +	10	Ach: 0.02 Ch: 1	Rat and mouse brain MD	II
Ach	LC	C-18 (2.1×150 mm, 5 µm)	20 mM NH_4Ac, 20 mM HFBA, MeOH	-	ESI+	50	Ach: 0.03	Rat brain MD	[63]
Ach	LC	DuetC18/SCX (2.1×150 mm)	32 mM NH_4Ac+ 25 mM NH_4form, pH 4 ACN	-	ESI+	10	Ach: 0.1	Rat brain MD	[73]

SCX = strong-cation-exchange
HFBA = heptafluorobutyric acid
APSI = atmospheric pressure spray ionization
Iso-Ach = (3-carboxypropyl)-trimethylammonium

Biogenic amines

Analytes	Separation	Column	Eluents	Derivati-zation	Ionization	Inj. [µl]	LOD (nM)	Sample	Ref
DA, NA, 5-HT	LC	C-18 (2×150 mm, 4 µm)	0.1% HCOOH ACN	-	ESI +	10	DA: 0.2 NA: 0.6 5-HT: 1	Rat brain MD	[93]
DA, 5-HT, 5-HIAA, HVA, DOPAC	LC	C-18 (2.1×150 mm, 5 µm)	0.1% HCOOH ACN	-	ESI+ / -	100		Rat brain	[105]
DA, 5-HT, NA, NM	LC	BEH phenyl (2.1×100 mm, 1.7 µm)	20 mM NH_4Ac 0.2% HCOOH MeOH	Acetalde-hyde-d_4	ESI +	20	DA: 33 pM NA: 30 pM 5-HT: 28 pM	Rat brain MD	[99]
NA, DA, L-Tyr	CapLC	PGC (0.2×150 mm, 5µm)	50 mM NH_4form MeOH	-	ESI +	2.5	NA: 3 DA: 10 Tyr: 30	Rat brain	[106]
DA, DA-G, DA-3-S, DA-4-S, NA, A, HVA DOPAC	LC	PFP (4×150 mm, 3 µm)	0.1% HCOOH ACN	-	ESI + / -	100	DA: 0.25 NA: 5 DOPAC: 10 HVA: 30 DA-G: 0.25 DA-3-S: 0.8 DA-4-S: 2	Rat and mouse brain MD	III
5-HT, 5-HIAA, DOPAC, HVA and their sulfates and glucuronides	LC	PFP (4×150 mm, 3 µm)	0.1% HCOOH ACN:MeOH	-	ESI + / -	100	5-HT: 0.1 5-HT-G: 0.1 5-HIAA: 1 5-HIAA-S: 4 DOPAC-S: 1 HVA-S: 0.5	Rat brain MD	IV

NM = normetadrenaline
PGC = porous graphitic carbon
PFP = pentafluorophenylpropyl

2. AIMS OF THE STUDY

The aim of the research was to develop sensitive and specific LC-MS/MS methods for the analysis of neurotransmitters in rat and mouse brain microdialysates and examine their glucuronidation and sulfation in the brain.

The more detailed aims of the research papers (I–IV) were:

- To develop and validate quantitative LC-MS/MS methods for the analysis of brain microdialysates (I–IV)
- To synthesize glucuronide and sulfate standards for method development (III, IV)
- To develop direct LC-MS/MS methods for the analysis of intact glucuronide and sulfate conjugates (III, IV).
- To study glucuronidation and sulfatation of neurotransmitters (DA, 5-HT) and their phase I metabolites (HVA, DOPAC, 5-HIAA) in the brain (III, IV).
- To compare the effect of different derivatization reagents on the ESI efficiency of amino acids (I).

3. MATERIALS AND METHODS

The chemicals, samples, instrumentation and analytical methods used in this work are briefly presented in this section. More detailed descriptions can be found in the original publications (I–IV).

3.1 Chemicals

All the chemicals used in this study were analytical or chromatographic grade. The structures of the compounds studied are shown in Figs 1–3, 5.

3.2 Derivatization of amino acids

The microdialysates and amino acid standards diluted in methanol (MeOH):H_2O (1:1) or Ringer's solution were evaporated to dryness (I). For propyl chloroformate (PrCF) derivatizations, the samples were dissolved in 80 µl of 0.1 M aqueous boric acid ($B(OH)_3$) (pH 8), and 40 µl of a mixture of propanol and pyridine (4:1, v/v) and 5–10 µl of PrCF were added. The samples were shaken, evaporated to dryness and dissolved in eluent for LC-MS/MS analysis.

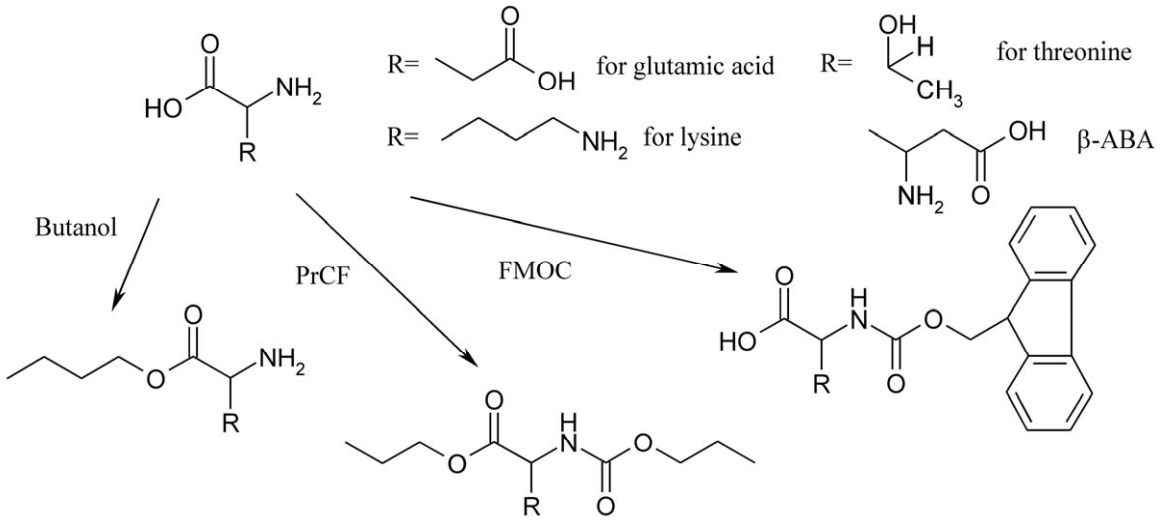

Figure 5. *Structure of derivatization products formed when test amino acids (Glu, β-ABA, Lys and Thr) were derivatized with butanol, FMOC or PrCF (I).*

Derivatization with 9-fluorenylmethyl chloroformate (FMOC) was performed by adding 100 µl of 0.1 M aqueous $B(OH)_3$ (pH 8) and 100 µl of 12 mM FMOC (in ACN) to the evaporated amino acid standards. The samples were shaken and the reaction was

performed at room temperature (25 °C) for 1 h. The samples were diluted to a final concentration of 30% ACN.

Derivatization with butanol was performed by adding 200 µl of a mixture of butan-1-ol and acetyl chloride (4:1, v/v) to the evaporated amino acid standards. The samples were shaken and the reaction was performed at 70 °C for 1 h. The samples were then evaporated to dryness and dissolved in eluent for LC-MS/MS analysis.

3.3 Synthesis of reference compounds

The glucuronide and sulfate conjugates of the neurotransmitters and their phase I metabolites were synthesized enzymatically or chemically for standard compounds to be used in the development of LC-MS/MS methods.

3.3.1 Enzymatic synthesis of glucuronide conjugates

The enzymatic synthesis of DA-, 5-HT-, 5-HIAA-, DOPAC- and HVA-Gs were performed, using microsomes prepared from rat liver (male Sprague-Dawley rats induced by Aroclor 1254 (a mixture of polychlorinated biphenyls)), as described previously [107]. The treatment of the rats was approved by the local Ethical Committee for Animal Studies. The protein concentrations of the microsomes were determined with the BCA Protein Assay Kit (Pierce Chemical, Rockford, IL, USA). In addition to the rat liver microsomes (protein concentration 1 mg/ml), the incubation mixture contained 2 mM DA, 5-HT, 5-HIAA, DOPAC or HVA, 5 mM saccharic acid 1,4-lactone, 5 mM UDPGA, 5 mM $MgCl_2$, 50 mM phosphate buffer (pH 7.4) and 2% ACN in a total volume of 30 ml. After incubation (37 °C) the reaction mixture was centrifuged and the supernatants were purified by solid-phase extraction (SPE). The glucuronides were fractionated by LC-UV and lyophilized, as described in more detail in the original publications (III, IV).

3.3.2 Enzymatic synthesis of sulfate conjugates

The incubation mixture contained 2 mM 5-HT, 5-HIAA, DOPAC, or HVA, 100 µM PAPS, 10 mM phosphate buffer (pH 7.4) and 30% v/v rat liver S9 fraction (male Sprague-Dawley rats induced by Aroclor 1254) as described previously [107]. The reaction mixture was incubated (37 °C) for 2 h and 5% aqueous $ZnSO_4$ was added (1:1, v/v). MeOH was added to a final concentration of 50%. After centrifugation the supernatant was purified by SPE and the solvent was evaporated by lyophilization. The DOPAC-Ss and HVA-S were fractionated by LC and the concentrations of the solutions were determined indirectly by determining the concentration of aglycone after acid hydrolysis by LC-MS/MS. The synthesis procedure is described in more detail in the original publication (IV).

3.3.3 Chemical synthesis of sulfate conjugates

The chemical synthesis of HVA-S, DOPAC-Ss, 5-HIAA-S and 5-HT-S was done, using concentrated H_2SO_4 as described previously for DA-S [108]. The reaction mixtures were neutralized with 5M NaOH and the sulfates were fractionated by LC-UV, evaporated to dryness and lyophilized (IV).

3.4 Analytical methods

The analyses were performed with an Agilent HP 1100 liquid chromatograph (Hewlett-Packard GmbH, Waldbronn, Germany) and a PE Sciex API3000 triple-quadrupole mass spectrometer (Applied Biosystems/MDS Sciex, Concord, Canada) or an Agilent 6410 triple-quadrupole mass spectrometer (Agilent Technologies, Santa Clara, USA). The more detailed conditions in each analysis are listed in Table 2 and the original publications (I–IV).

3.5 Microdialysis samples

Male and female mice or male Wistar rats were used at 8–16 weeks of age. All procedures with animals were performed according to the European Community Guidelines for the Use of Experimental Animals (European Communities Council Directive 86/609/EEC), reviewed by the State Provincial Office of Southern Finland and approved by the Animal Experiment Board in conformance with current legislation.

The animals were implanted with guide cannulae, using a stereotaxic device (Stoelting, Wood Dale, IL, USA) under isoflurane anaesthesia. In rats, the guide cannula was aimed at the nucleus accumbens, above the rat dorsal striatum or the medial prefrontal cortex according to the atlas by Paxinos and Watson [109]. For mice the guide cannula was aimed at a point above the striatum or nucleus accumbens according to the mouse brain atlas [110]. The cannula was fastened to the skull with dental cement (Aqualox, Voco, Germany) and stainless-steel screws. Buprenorphine was given for postoperative pain. The animals were allowed to recover for 5–7 d before the experiment.

The microdialysis probe was inserted into the guide cannula of the animal the day before or on the morning of the experimental day. The probe was infused with Ringer's solution at a flow rate of 2–2.5 µl/min. The Ringer's solution contained 147 mM NaCl, 1.2 mM $CaCl_2 \cdot 2\ H_2O$ (Merck, Darmstadt, Germany), 2.7 mM KCl (Riedel-deHaën, Seelze, Germany), 1.0 mM $MgCl_2 \cdot 6\ H_2O$ (Merck, Darmstadt, Germany), and 0.04 mM ascorbic acid (University Pharmacy, Helsinki, Finland) and was also used for the dilution of standards. The microdialysis samples were stored in a freezer (-70 °C) before analysis with LC-MS/MS.

Table 2. *Analysis conditions used in the study of different neurotransmitters by LC-MS/MS. The qualifier ions are shown in parentheses.*

Analytes	SRM pairs (*m/z*)	Gradient Time, min	Org %	Chromatographic conditions	Ref.
PrCF deriv.					I
Arg	303 → 156 (70)	0 - 10	10 - 70	Atlantis™ dC-18	
Asn	261 → 130 (216)	10 - 12	70	(50×2.1 mm, 3 µm)	
Ser	234 → 146 (174)			0.1% aqueous HCOOH	
Gly	204 → 102 (144)			ACN	
Thr	248 → 160 (74)			Injection volume 15 µl	
Ala	218 → 130 (158)				
α-ABA	232 → 144 (102)				
β-ABA	232 → 172 (130)				
GABA	232 → 172 (130)				
Glu	318 → 172 (258)				
Lys	361 → 170 (241)				
FMOC deriv.					
β-ABA	326 → 104 (179)	0 - 10	30 - 80		
Glu	370 → 130 (179)	10 - 12	80		
Lys	591 → 369 (179)				
Thr	342 → 120 (179)				
BuOH deriv.					
β-ABA	160 → 104 (44)	0 - 10	10 - 70		
Glu	260 → 130 (84)	10 - 12	70		
Lys	203 → 84 (130)				
Thr	176 → 102 (74)				
Ach	146 → 87	0 - 3	80	Lichrospher diol	II
Ch	104 → 104	3 - 3.8	80 - 65	(125 × 4 mm, 5 µm)	
A-β-MeCh	160 → 101	3.8 - 5	65 - 50	20 mM NH_4formate, pH 3.3, ACN. Injection volume 10 µl.	
		5 - 7.5	50		
DA	154 → 137 (91, 65)	0 - 10	5 - 35	Discovery HSF5	III
DA-S	232 → 152 (122, 80)	10 - 10.1	35 - 80	(150 × 4 mm, 3 µm)	
DA-G	330 → 137 (154, 91)	10.1 - 13	80	0.1% aqueous HCOOH	
NA	170 → 135 (107)			ACN	
A	184 → 107 (77)			Injection volume 100 µl	
DOPAC	167 → 123 (95)				
HVA	181 → 122 (74)				
5-HT	177 → 160 (132, 117, 115)	0 - 1	5	Discovery HSF5	IV
5-HT-G	353 → 160 (336, 177)	1 - 11	5 - 20	(150 × 4 mm, 3 µm)	
5-HT-S	257 → 115, 160, 240	11 - 13	20 -65	0.1% aqueous HCOOH	
DA	154 → 137 (91)	13 - 15	65	ACN:MeOH (1:1)	
DA-G	330 → 137 (154, 91)			Injection volume 100 µl	
5-HIAA	190 → 146 (144, 116)	0 - 1	5		
5-HIAA-S	270 → 226 (146, 131, 80)	1 - 11	5 - 20		
5-HIAA-G	366 → 113, 146	11 - 13	20 - 85		
DOPAC	167 → 123 (108, 95, 93)	13 - 15	85		
DOPAC-G	343 → 113, 123				
DOPAC-S	247 → 123 (203, 167, 80)				
HVA	181 → 137 (122)				
HVA-G	357 → 113, 123				
HVA-S	261 → 181 (217, 80)				

4. RESULTS AND DISCUSSION

The main results obtained in this work are described shortly in this section. More detailed information can be found in the original publications (I–IV).

The concentrations of neurotransmitters and their metabolites in brain microdialysates are low (fmol amounts) and the sample volume is limited (typically < 40 µl). Many other endogenous compounds in addition to neurotransmitters are also extracted from the brain by microdialysis, and therefore the analytical method must be sensitive and specific. The perfusion fluid used in the microdialysis contains a high concentration of inorganic salts (150 mM), whereas the concentrations of small polar neurotransmitters are low, making demands on chromatographic separation. LC-MS/MS provides sufficient specificity and sensitivity for the analysis of neurotransmitters, but the inorganic salts must be separated from the compounds studied to provide sufficient stability and repeatability. The MS detection is not widely used in the analysis of neurotransmitters in the brain (Table 1) and the aim of this study was to develop LC-MS/MS methods for the analysis of different neurotransmitters and their metabolites in brain microdialysates.

4.1 Analysis of amino acids

The retention of polar amino acids on reversed-phase columns is poor and therefore ion-pair chromatography has often been used in the analysis of amino acids by LC-MS/MS [111-113]. However, the ion-pair reagents decrease the ionization efficiency of the amino acids in ESI due to ion suppression, complicating particularly the quantitative analysis of GABA, whose concentration in brain microdialysate is low: 9–170 nM [47, 49, 52, 55, 56, 58, 59]. The limit of quantification (LOQ) for GABA, using ion-pair chromatography connected to MS/MS was too high (100 nM, 0.5 pmol [113]) for the analysis of brain microdialysates. Amino acids have also been analysed with HILIC, which allows the use of nonsuppressive and more MS-compatible eluents, such as volatile aqueous buffers and ACN [114, 115], but the LODs for GABA were still too high for the quantitative analysis of brain microdialysates (5 µM, 50 pmol [115] or 20 nM, 6 fmol [62]). ESI sensitivity is not optimal for small and polar compounds, such as amino acids. It is known that the nonpolar properties of the compound increase the fraction of the analyte on the surface of the droplet formed in the ESI process [116]. The polarity of amino acids can be decreased by derivatization, thereby improving their ionization efficiency. The interaction of more hydrophobic derivatives with a reversed-phase stationary phase is stronger and they are eluted at higher organic content, which also increases the ESI sensitivity due to lower surface tension, higher volatility and less efficient solvation of ions compared with highly aqueous eluents [117]. Separation of the signal-suppressing inorganic salts of Ringer's solution from amino acid derivatives in reversed-phase chromatography is also easier than from native amino acids under similar conditions.

4.1.1 Comparison of different derivatization reagents

Three commonly used derivatization reagents (FMOC, butanol and PrCF) were compared in the quantitative LC-MS/MS analysis of amino acids (I). Since FMOC reacts with the amino group, butanol with the carboxylic acid group and PrCF with the amino, hydroxyl and carboxylic acid groups, amino acids with different chemical properties, such as basic lysine (Lys), acidic Glu, neutral β-aminobutyric acid (β-ABA) and threonine (Thr) containing a hydroxyl group, were used as test compounds (Fig. 5).

The reaction conditions were optimized to produce the maximal amounts of derivatization products. Different buffer systems were tested to optimize the PrCF and FMOC derivatization procedures (Fig 6). When FMOC was used, the amount of derivatization products was highest using 0.1 M aqueous $B(OH)_3$, at pH 8. Acidic solvents were also tested (0.03 M hydrochloric acid (HCl) and 0.1% aqueous HCOOH), but the FMOC derivatization of amino acids was minimal and the derivatization products were barely detectable. The aqueous $B(OH)_3$, aqueous ammonia (NH_3) (pH 9.8) and 0.03 M HCl were advantageous in the derivatization with PrCF, with $B(OH)_3$ working best. Different temperatures (70, 110 °C) and reaction times (15–60 min) were tested in the derivatization with butanol and the best results were obtained when the reaction was performed at 70 °C for 1 h.

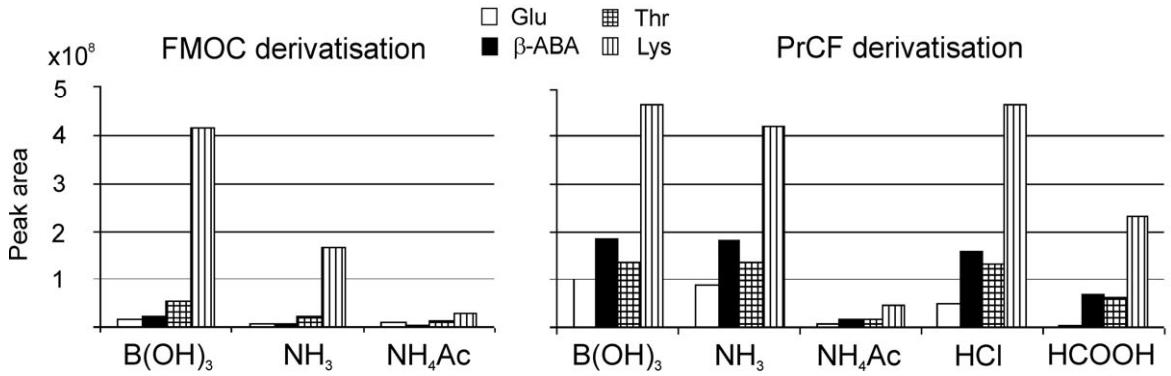

Figure 6. *Comparison of different solvents in derivatization efficiency of Glu, β-ABA, Thr and Lys by FMOC and PrCF: 0.1 M aqueous boric acid, pH 8 ($B(OH)_3$), aqueous ammonia, pH 9.8 (NH_3), 0.1 M aqueous ammonium acetate (NH_4Ac), 0.03 M HCl and 0.1% aqueous formic acid (HCOOH).The derivatives were analysed with LC-MS and the peak areas of the main products are shown.*

The derivatization reactions with butanol, FMOC and PrCF were not complete and partially derivatized products, in which not all the possible functional groups reacted with the reagent, were formed. Since PrCF can react with amino, carboxylic acid and hydroxyl groups, from two to four different products were formed, due to incomplete derivatization reactions (Fig. 7). Since FMOC and butanol react only with the amino or carboxylic acid groups, respectively, the number of products formed was smaller than with PrCF (one or two). Of amino acids tested, only acidic Glu in the reaction with butanol and basic Lys in

the reaction with FMOC can form more than one derivatization product. The relative amount of fully reacted butanol-derivatized Glu was 90% and fully reacted FMOC-derivatized Lys 39%. For all FMOC-derivatized amino acids, a side product having a molecular weight one mass unit lower than that of the respective fully reacted main product was formed. These side products (relative amount 10–33%) were baseline-separated from the main products and did not disturb the analysis of the main product. When PrCF was used the main products for β-ABA, Lys and Glu were fully reacted amino acids (relative amount 50–98%), in which all the reactive functional groups were derivatized (Fig. 7). The derivatization of the hydroxyl group of Thr by PrCF was not favourable and in the main product only the amino and carboxylic acid groups were derivatized (relative amount 85%). The relative amounts of other products formed in the PrCF derivatizations are shown in Fig. 7. Even though many products were formed the repeatability of the PrCF derivatization procedure was good, the %RSD being 5–7% (n = 6, 800 nM) for the main product (Table 3). The repeatability was 1–7% and 5–13% for the main products derivatized with butanol and FMOC, respectively, which was acceptable for bioanalysis and indicated that the repeatability of the derivatization step is good for all the derivatization reagents.

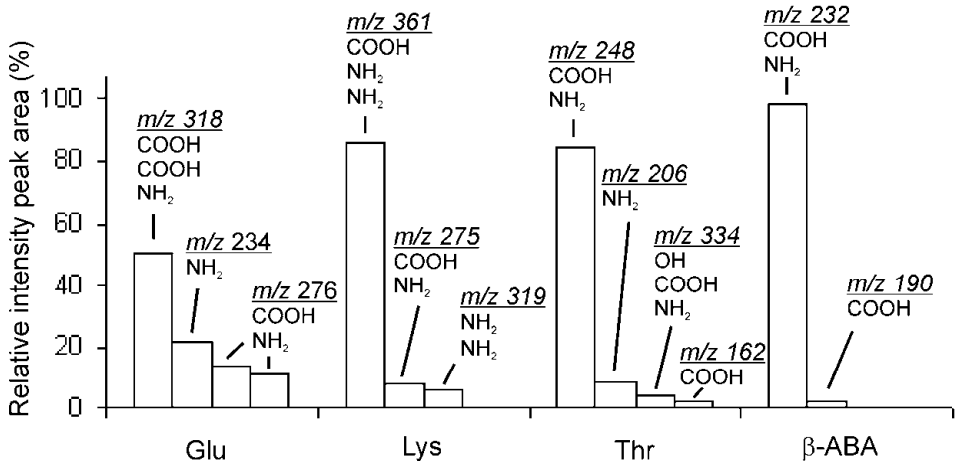

Figure 7. *Relative amounts of different products formed in the derivatization of 5 µM Glu, Lys, Thr and β-ABA with PrCF. The derivatives were analysed with LC-MS in selected ion-monitoring mode. The m/z of the protonated products and functional groups of the amino acids, that reacted with PrCF are shown.*

The Ringer's solution used in brain microdialysis contains high amounts of inorganic salts (>150 mM) that may affect the derivatization of amino acids. The effect of high salt concentration on the yields of the derivatization products was tested with the derivatization of the test amino acids (800 nM) diluted in Ringer's solution (Table 3). The comparison showed that high salt concentrations decreased the yields of the butanol derivatives (Glu, β-ABA and Thr) by 4–15% and FMOC-derivatized Lys by 28%. The yields of the PrCF derivatives increased by 3–22% and the FMOC derivatives other than Lys by 0–46%. The results indicate that the derivatization procedures can also be applied at high salt concentrations. The repeatabilities were slightly higher for amino acids

derivatised in Ringer's solution than in MeOH:H_2O (1:1), (Table 3) but they were less than 15% (except 65% for FMOC-derivatised Lys), which is acceptable for a bioanalytical method.

The collision-induced dissociation of four differently derivatized test amino acids was studied (Table 4, I). The main product ion in the MS/MS spectra of all FMOC derivatives was at *m/z* of 179 $[M+H-CO_2\text{-amino acid}]^+$. Cleavage of the FMOC moiety (loss of 222 Da), producing a protonated amino acid, was also common (intensity 31–39%), and the fragmentation continued with the loss of water for the β-ABA, Glu, and Thr derivatives. The loss of 1-butene, commonly occurring together with the loss of water (β-ABA, Glu and Thr) or NH_3 (β-ABA and Lys), was observed in the MS/MS spectra of protonated molecules of butanol-derivatized amino acids. The fragmentation patterns occurring after the loss of 1-butene were the same as those observed for underivatized amino acids [112, 118]. The MS/MS spectra of the PrCF derivatives showed more product ions than those of the other derivatives. The loss of propanol (60 Da) was common for all PrCF-derivatized amino acids, and the fragmentation continued with the loss of carbon monoxide (Glu, Lys and Thr) or water (Thr). The loss of 1-propene was also observed in the MS/MS spectra of protonated molecules of PrCF-derivatized amino acids.

The retention of the PrCF and FMOC derivatives of the amino acids in reversed-phase chromatography was better than that of the butanol derivatives (Figure 2, I). The derivatization with butanol does not mask the amine, which is protonated under the chromatographic conditions used and reduces the retention of the derivatives. The ionization efficiency of the amino acids was significantly improved after derivatization (2–60 times) compared with that of native amino acids. In analysis of the derivatized amino acids, good repeatability (%RSD < 9%) and low LODs (0.5–2 nM, 7.5–30 fmol, signal-to-noise ratio (S/N) of at least 3) were observed with the PrCF and butanol derivatives (Table 3). The repeatabilities were similar to those of other studies in which the butanol and chloroformate derivatives of the amino acids were analysed with LC-ESI-MS/MS [119, 120]. Usually the criterion for a good calibration curve is that the concentration of standard should not deviate from the nominal concentration by more than 15% except at LOQ 20% deviation is accepted. The linearity did not meet the criteria at the lower concentration range (10-100 nM) with butanol derivatised amino acids and PrCF derivatised Thr, deviation <35% (Table 3). Better linearity was observed at the higher concentration range (0.5-8 µM) for butanol and PrCF derivatised amino acids, the deviation being less than 16%. Derivatization with FMOC was less suitable for the quantitative analysis of amino acids, due to the method's poorer linearity (r^2 = 0.925–0.994, deviation <59%, Table 3), repeatability (%RSD < 13%) and slightly higher LODs (0.5–5 nM, 7.5–75 fmol) than those achieved with the other reagents. Peaks at the rts of the derivatized amino acids (S/N = 2-9) were observed in the analysis of the FMOC-, PrCF- and butanol-derivatized blank samples, complicating the analysis of amino acids at low concentrations. These memory effects in the analysis of amino acids in microdialysis samples have also been reported previously after OPA derivatization by LC using amperometric detection [46, 52] and after NDA derivatization by LC-FL [51].

Table 3. *Validation results of the LC-MS/MS method for the analysis of derivatized test-amino acids.*

Derivatization	Parameter	β-ABA	Glu	Lys	Thr
PrCF	Retention time (min)	10.1	11.9	11.5	8.4
	SRM pair, *m/z*	232 → 172	318 → 172	361 → 241	248 → 160
	r^2 (10-100 nM) (Accuracy)	0.978 (85–109%)	0.990 (87–107%)	0.992 (85–108%)	0.981 (83–112%)
	r^2 (0.5-8 µM) (Accuracy)	0.999 (86–116%)	0.999 (90–107%)	0.999 (93–108%)	0.999 (96–107%)
	Intra-assay precision, %RSD (800 nM, n=6)	5.2	7.0	5.4	6.6
	LOD (nM, S/N = 3)	0.5 (7.5 fmol)	2 (30 fmol)	0.7 (10.5 fmol)	1 (15 fmol)
	Relative intensity in Ringer's solution[a]	103%	117%	103%	122%
	Intra-assay precision, %RSD (800 nM, n=6) in Ringer	9.9	10.5	8.3	6.0
FMOC	Retention time (min)	7.4	6.0	11.5	6.2
	SRM pair, *m/z*	326 → 104	370 → 130	591 → 369	342 → 179
	r^2 (10-100 nM) (Accuracy)	0.981 (85–134%)	0.982 (93–134%)	0.943 (85–136%)	0.963 (89–129%)
	r^2 (0.5-8 µM) (Accuracy)	0.972 (41–144%)	0.979 (73–136%)	0.925 (56–136%)	0.994 (73–123%)
	Intra-assay precision, %RSD (800 nM, n=6)	7.9	11.4	5.3	13.2
	LOD (nM, S/N = 3)	4 (60 fmol)	5 (75 fmol)	0.5 (7.5 fmol)	1 (15 fmol)
	Relative intensity in Ringer's solution[a]	100%	146%	72%	115%
	Intra-assay precision, %RSD (800 nM, n=6) in Ringer	15.3	11.5	65.1	6.6
Butanol	Retention time (min)	3.0	7.9	0.9	1.7
	SRM pair, *m/z*	160 → 104	260 → 130	203 → 84	176 → 74
	r^2 (10-100 nM) (Accuracy)	0.994 (85–125%)	0.989 (86–135%)	0.986 (85–121%)	0.993 (90–127%)
	r^2 (0.5-8 µM) (Accuracy)	0.999 (92–104%)	0.998 (90–107%)	0.996 (92–108%)	0.999 (91–110%)
	Intra-assay precision, %RSD (800 nM, n=6)	1.8	3.7	1.4	7.4
	LOD (nM, S/N = 3)	1 (15 fmol)	1 (15 fmol)	1 (15 fmol)	1 (15 fmol)
	Relative intensity in Ringer's solution[a]	96%	89%	-[b]	85%
	Intra-assay precision, %RSD (800 nM, n=6) in Ringer	7.0	4.5	-[b]	7.7

[a] The relative intensity of amino acids derivatized in Ringer's solution compared with amino acids derivatized in MeOH:H_2O (1:1).

[b] Butanol-derivatized Lys coeluted with the salts of Ringer's solution and was directed to waste.

In the analysis of amino acids better repeatability, linearity and lower LODs were observed with the PrCF and butanol derivatives than for the FMOC derivatives. The separation of amino acids from the inorganic salts of Ringer's solution is necessary, to avoid suppression effects in ESI, and this was properly achieved with the FMOC and PrCF derivatives, but not with the butanol derivatives. Based on the results obtained with the four test amino acids, PrCF seemed, of the reagents tested, to be the best derivatization agent for the analysis of amino acids in rat brain microdialysates.

4.1.2 Rat brain microdialysates

Non-specific analytical methods, such as LC-EC [47, 52], LC-FL [58] and CE-LIF [49, 55, 56, 59], have commonly been used for the analysis of amino acids in brain microdialysates. A more specific LC-ESI-MS/MS technique has been used in only a few studies: for the analysis of amino acids sampled from monkey brain [62] and D- and L-serine in rat brain microdialysates [103]. However, analysis of GABA in rat brain microdialysates by LC-MS/MS has not been presented in the literature.

The suppression effect of the sample matrix on the signal intensity of the amino acid derivatives was studied by injecting a PrCF-derivatized rat brain microdialysis sample into the column and following the intensity of PrCF-derivatized β-Alanine (β-Ala), which was continuously infused and combined with the LC effluent through a T-piece after the analytical column (Fig. 3, I). The intensity of the β-Ala signal was similar, whether the injected sample was a solvent, a PrCF-derivatized microdialysis sample or a Ringer's solution blank, indicating that the microdialysate contained no signal-suppressing components. The rat brain microdialysates were spiked with 1000 nM arginine (Arg), asparagine (Asn), Ser, Gly, Thr, Ala, 100 nM Glu and 50 nM GABA, α-ABA and β-ABA. The recovery for the addition was acceptable for all amino acids tested, being between 79% and 120% (Table 2, I), also indicating that no suppressing compounds were coeluting with the amino acids studied. The concentration of amino acids deviated from the added amount less than 15% (except of Arg (21%) and Ser (20%)), which is considered as acceptable accuracy for a bioanalytical method. Calibration curves for each amino acid were measured, and the linearity was acceptable, the r^2 being 0.9851–0.9996 and deviation less than 15%, except in one data point for Asn (49%), Thr (23%) and Glu (29%).

In all, ten amino acids were analysed in rat brain microdialysates collected from the striatum by LC-MS/MS after precolumn PrCF derivatization (Fig. 8). In fact, this was the first time that GABA, the predominant inhibitory neurotransmitter in the brain, was analysed in rat brain microdialysates by LC-MS/MS. Seven microdialysis samples were collected at 30 min intervals and GABA was analysed in parallel with specific LC-MS/MS (PrCF derivatization) (I) and LC-FL (OPA derivatization) [54]. The concentrations of GABA were 12–26 nM (rat 1) and 37–63 nM (rat 2) with both methods, indicating that GABA can be accurately quantified with LC-MS/MS and LC-FL (Table 5, I).

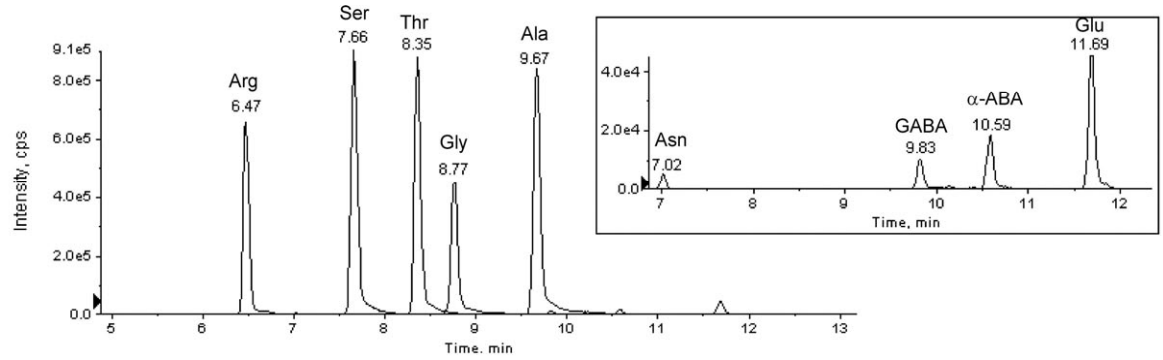

Figure 8. *LC-MS/MS analysis of rat brain microdialysate derivatized with PrCF. The SRM pairs followed for each amino acid were m/z 303→156 for Arg, m/z 261→130 for Asn, m/z 234→146 for Ser, m/z 248→160 for Thr, m/z 204→102 for Gly, m/z 218→130 for Ala, m/z 232→172 for GABA and β-ABA, m/z 232→144 for α-ABA and m/z 318→172 for Glu (I).*

GABA and its isomers α- and β-ABA (also extracted from the brain) were chromatographically separated from each other and the isomers did not interfere with the quantitative LC-MS/MS analysis of GABA in the microdialysates (Fig 9). Interestingly, the concentration of α-ABA was similar to that of GABA. The concentrations of amino acids other than the ABA isomers in brain microdialysates were 0.2–9 µM (Table 5, I). The concentration of Glu (0.2–0.6 µM) was lower than in other studies using CE-LIF (3–11 µM [49, 59]) but the flow rate of the perfusion fluid, which affects the recovery of amino acids, was higher in our study (2 µl/min versus 1 µl/min [49, 59]).

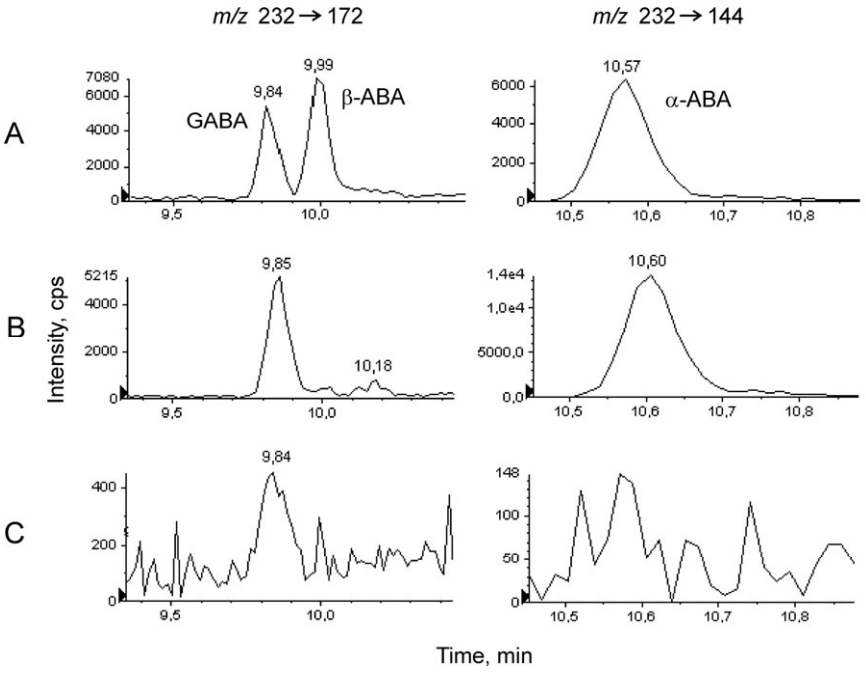

Figure 9. *Analysis of PrCF-derivatized GABA, β-ABA and α-ABA by LC-MS/MS in standards diluted in Ringer's solution (a) and rat brain microdialysates (b). SRM chromatogram of Ringer's solution (c, blank).*

4.2 Analysis of acetylcholine and choline

Derivatization of amino acids was used to improve their retention in reversed-phase chromatography and ESI efficiency (I). Instead of derivatization, good retention for native neurotransmitters can be achieved using polar stationary phase, such as diol, silica or amine, for which polar compounds have high affinity. In HILIC the retention of polar compounds is increased when the proportion of organic solvent is increased and aqueous mobile phases usually containing more than 50% organic solvent are used. The high organic content in the eluent is also favourable for the sensitive ESI of analytes. HILIC was employed in the analysis of ionic Ach and Ch, which have minimal retention in reversed-phase chromatography (II).

4.2.1 HILIC-MS/MS method development

A diol column was used for the LC-MS/MS analysis of Ach and Ch in brain microdialysates (II). The analysis was carried out using 80% ACN in the eluent. The buffer concentration (10, 20 or 30 mM aqueous ammonium formate) clearly affected the elution of chloride ions in Ringer's solution (measured as an ammonium chloride cluster at *m/z* 338 $[(NH_4Cl)_6NH_4]^+$) and also on the peak width of Ach and Ch (Fig. 10). The lower the buffer concentration, the broader was the elution band of chloride ions. The coelution of chloride ions with Ach and Ch increased the theoretical plate numbers of the analytes, but also caused ion suppression and decreased sensitivity. The best sensitivity was obtained under conditions in which the chloride ions were only slightly separated from Ach (20 mM buffer). The chloride ions coeluted with Ch under all separation conditions tested. The ion suppression caused by chloride ions in Ch ionization did not interfere with the quantitative analysis, since the concentration of Ch in brain microdialysates is high (at µM the level). However, the chloride ions caused corrosion of the stainless-steel union of the ion source. When the parts were changed to a more inert alternative (Hastelloy C®, nickel-chromium-molybdenum alloy), the analysis could be carried out for weeks without the clogging caused by corrosion. Clogging of the MS interface was previously reported when microdialysis samples were analysed with LC/MS [121]. The cations of Ringer's solution were well separated from the analytes and were washed into the waste after elution of Ch (II).

Due to the high organic content in the eluent (80%), low LODs (S/N = 3) of 0.02 and 1 nM (0.2 and 10 fmol) were achieved for Ach and Ch, respectively (II). Even lower LODs for Ach were achieved in later studies employing HILIC-MS/MS by decreasing the column diameter [62, 71]. LODs of 0.05 nM (0.015 fmol) [62] and 0.0075 nM (0.075 fmol) [71] for Ach were reported, using 0.2 and 2.1 mm internal diameter (i.d.) HILIC columns, respectively. Low LODs of 0.01 nM (0.2 fmol, column i.d. 2.1 mm) [70] and 0.04 nM (0.012 fmol, colum i.d. 0.075 mm) [72] were also achieved, using smaller diameter cation exchange columns. Even though Ringer's solution is a strong solvent in HILIC, the brain microdialysates could be injected as such in this study (II). In other

HILIC separation systems, dilution of the samples with organic solvent was needed [62, 71]. The between-day repeatability (%RSD 3.1–3.5%) and linearity of the method was good, with r of 0.999 (0.1–50 nM, deviation <5%) and 0.998 (0.1–3.5 µM, deviation <15%) for Ach and Ch, respectively (II).

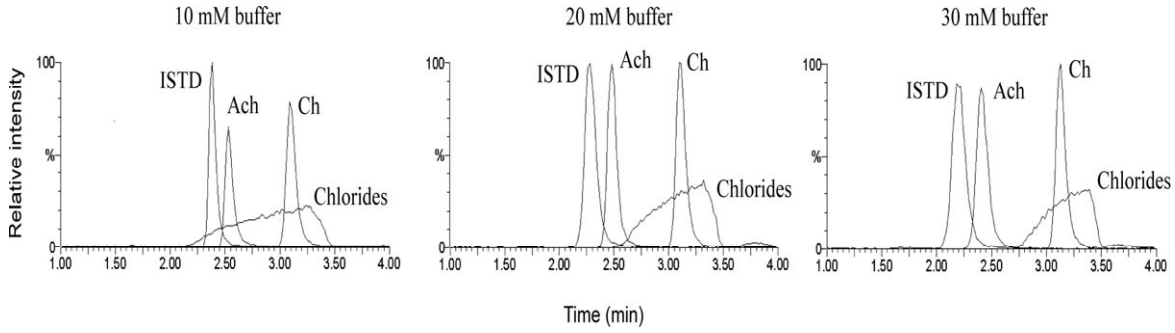

Figure 10. *LC-MS extracted ion chromatograms of 1 nM Ach (m/z 146), 500 nM Ch (m/z 104), 5 nM ISTD (m/z 160) and chlorides (m/z 338 $[(NH_4Cl)_6NH_4]^+$) measured using 10, 20 and 30 mM ammonium formate (pH 3.3) buffer : ACN (20:80) (II).*

4.2.2 Rat and mouse brain microdialysates

In the analysis of Ach and Ch, acetyl-β-methylcholine was used as an ISTD (II). It is known that endogenous compounds with the same mass transitions as Ach (*m/z* 146→87) and ISTD (*m/z* 160→101) are coextracted from the mouse brain [63, 70]. Both of the endogenous compounds were well separated from Ach and the ISTD and did not disturb the analysis in our study (Fig. 11). The concentration of Ach in brain microdialysates is low, and therefore blank samples (pure Ringer's solution) were analysed to study a possible carry-over effect. The selected reaction monitoring (SRM) chromatogram (*m/z* 146→87) of a blank sample showed no signals at the rt of Ach. However, a peak (S/N = 2.5) at the rt of Ch was observed in the SRM chromatogram (*m/z* 104→104), but the peak height of Ch in brain microdialysate was much higher (500 times) and the quantitative analysis was not compromised. The rat brain microdialysates were spiked with 1 or 2.5 nM Ach and 0.2 or 1 µM Ch. The recovery for the addition was acceptable (90–101%), indicating that no suppressing compounds were coeluting with Ach and Ch and accuracy of the method is good. We observed that Ch decomposed totally in the brain microdialysate samples stored at room temperature for 24 h, while it was stable for at least three days when stored in the freezer (-20 °C). However, no decomposition of Ch diluted in Ringer's solution and stored at room temperature for three days was observed. These results indicate that the microdialysis samples should be analysed or frozen immediately after collection and thawed just before the analysis. Ach was stable for 72 h, whether the microdialysis sample was stored at room temperature or in the freezer.

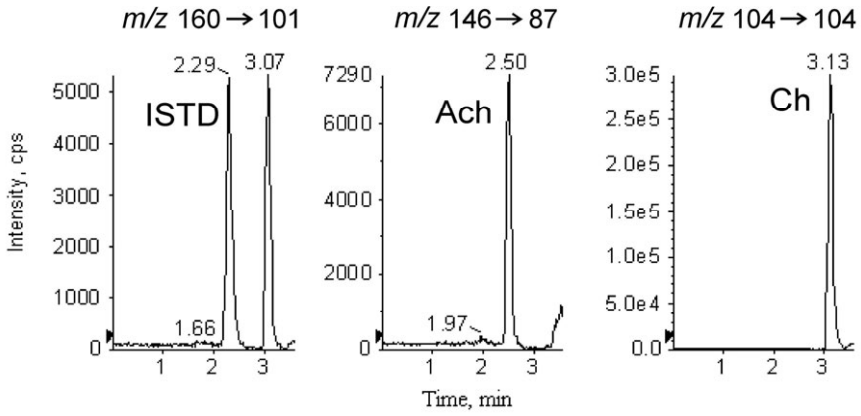

Figure 11. *SRM chromatograms of a mouse brain microdialysate collected from the nucleus accumbens with an acetyl-β-methylcholine ISTD addition.*

This was the first time that HILIC-MS/MS was employed in the analysis of Ach and Ch in brain microdialysates (II). The concentrations measured for Ach in the rat and mouse brain microdialysates were at least eight times higher than the LOQ, indicating that the method can potentially be used for reliable analysis of Ach in brain microdialysis samples without the use of acetylcholinesterase inhibitors.

4.3 Analysis of biogenic amines and their phase I and II metabolites

Sulfation and glucuronidation of neurotransmitters and phase I metabolites in the brain has usually been studied by indirect analytical methods employing acidic or enzymatic hydrolysis. The indirect analytical methods, however, are prone to errors due to the hydrolysis step, which also complicates the analysis. Even though the type of conjugate can be investigated with greater certainty after specific enzymatic than after acidic hydrolysis, the presence of the conjugate is still difficult to confirm by indirect methods, especially if the concentration of conjugate is low compared with that of free aglycone. The differentiation of separate regioisomers of glucuronide and sulfate conjugates is also impossible with indirect methods employing hydrolysis. One reason for the use of indirect methods is the lack of commercial sulfate and glucuronide standards. The sulfate and glucuronide conjugates of DA, HVA, DOPAC, 5-HT and 5-HIAA were chemically and/or enzymatically synthesized to provide authentic reference compounds for a direct LC-MS/MS method (III, IV).

4.3.1 Enzymatic synthesis of glucuronides

The glucuronides of DA, 5-HT, 5-HIAA, HVA and DOPAC were synthesized enzymatically, using rat liver microsomes as a biocatalyst (III, IV). The chromatographic

separation of different regioisomers of the glucuronide conjugates was achieved with a pentafluorophenylpropyl (PFP) column. LC-MS extracted ion chromatograms of the deprotonated molecules showed one peak for 5-HT-G, two peaks for 5-HIAA-Gs, and four peaks for DOPAC-Gs and HVA-Gs. Two regioisomers of DA-Gs were produced, but the intensity of the minor glucuronide product was only 1% of the main product peak. Abundant $[M-H]^-$ corresponding to mono glucuronidated compounds was observed in the negative ion mass spectra of DA-G, 5-HT-G, 5-HIAA-Gs, HVA-Gs and DOPAC-Gs and $[M+H]^+$ in the positive ion mass spectra of DA-G and 5-HT-G. The respective negative ion MS/MS spectra showed product ions at *m/z* 175 $[M-H\text{-aglycone}]^-$, *m/z* 157 $[M-H\text{-aglycone-}H_2O]^-$ and *m/z* 113 $[M-H\text{-aglycone-}H_2O\text{-}CO_2]^-$ derived from the glucuronic acid moiety, confirming the presence of the glucuronic acid moiety in the molecule (Fig 12). The glucuronidation site of 5-HT-G and DA-G was determined by nuclear magnetic resonance (NMR) spectroscopy. Heteronuclear multiple bond correlation (HMBC) experiment showed 3J coupling of the acetal CH proton (H1') to the quaternary aromatic carbon ArCO (C5), indicating that the glucuronide group is attached to the phenolic oxygen of 5-HT. In a previous study it was also shown that the glucuronidation of 5-HT with mouse liver microsomes occurred at the phenol group [86]. The ^1H NMR analysis showed that the main product formed in the synthesis of DA-G was glucuronidated at the 4-position, because the chemical shift that was most strongly moved downfield in DA-G compared with DA was that of aromatic H5.

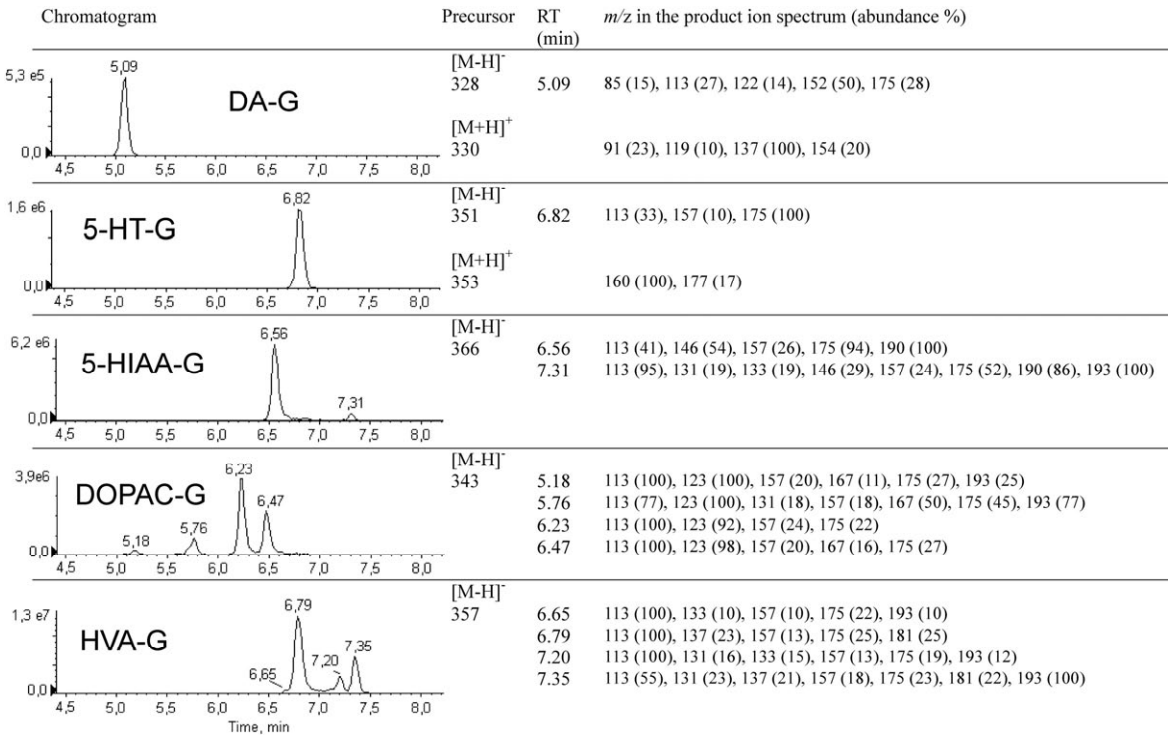

Figure 12. *LC-ESI-MS/MS analysis of enzymatically synthesized DA-G, 5-HT-G, 5-HIAA-G, DOPAC-G and HVA-G. The product ion chromatograms and most abundant (>10%) m/z values in the MS/MS spectrum of $[M-H]^-$ is shown. For 5-HT-G and DA-G, the m/z values in the product ion spectrum of $[M+H]^+$ are also shown (III, IV).Linear gradient 5–35% ACN:MeOH(2:1) for 0-10 min was used.*

The recovery for the glucuronides of the phase I metabolites (HVA, DOPAC and 5-HIAA) was too low to study their structure by NMR and therefore only negative ion MS/MS spectra were used for the identification. A product ion at *m/z* 193 (glucuronic acid) was observed in the MS/MS spectra of three HVA-Gs (rts 6.65, 7.20 and 7.35 min), two DOPAC-Gs (rts 5.18 and 5.76 min) and one 5-HIAA-G (rt 7.31 min) (Fig 12). It was previously reported that in the MS/MS spectra of aliphatic hydroxyl glucuronides, a product ion at *m/z* 193 is observed, whereas it is not observed in the MS/MS spectra of phenol-linked glucuronides [122-124]. Therefore it is reasonable to assume that those HVA-Gs, DOPAC-Gs and 5-HIAA-G showing a product ion at *m/z* 193 in the MS/MS spectra were glucuronidated to the carboxylic group, producing acyl glucuronides. The DOPAC-Gs (rts 6.23 and 6.47 min), HVA-G (rt 6.79 min) and 5-HIAA-G (rt 6.56 min) were most probably glucuronidated at the phenolic groups since *m/z* 193 was not observed in the MS/MS spectra. However, the glucuronidation site cannot be confirmed with certainty by MS/MS studies.

4.3.2 Enzymatic synthesis of sulfates

DOPAC-, HVA, 5-HT- and 5-HIAA-Ss were synthesized enzymatically, using the rat liver S9 fraction and analysed by LC-MS (IV). One monosulfated product was observed for 5-HT, 5-HIAA and HVA, while two monosulfated products were formed for DOPAC. The product ion spectra of 5-HT-S, DOPAC-Ss and HVA-S showed the loss of sulfur trioxide (80 Da), producing the free substrate and clearly indicating the presence of the sulfate moiety in the molecule (Fig. 13). The loss of substrate producing a product ion at *m/z* 80 was observed in the MS/MS spectra of 5-HT-S, 5-HIAA-S and HVA-S. The product ion spectrum of $[M-H]^-$ of 5-HIAA-S, DOPAC-Ss and HVA-S showed abundant product ions at *m/z* 226, *m/z* 203 and *m/z* 217, respectively, formed by the direct loss of carbon dioxide (44 Da) from the $[M-H]^-$ ion and indicating that sulfate is not attached to the carboxylic acid group of the substrate. It is known that the hydroxyl group is a common acceptor group of sulfate in the reaction catalysed by SULT [125], and therefore the most obvious sulfatation site is a phenolic hydroxy group. However, the yield of sulfates in the enzymatic synthesis was too low to study the conjugation site by NMR and therefore chemical synthesis was applied.

4.3.3 Chemical synthesis of sulfates

Chemical synthesis yielded one monosulfated main product for HVA and 5-HT, while two different monosulfated 5-HIAA-Ss and DOPAC-Ss were formed (Fig. 13). The two DOPAC-S isomers are almost coeluting in Fig. 13 (eluents ACN:MeOH (1:1), 0.1% aqueous HCOOH), but they were baseline-separated when a different eluent system was used (ACN, 0.1% aqueous HCOOH). Identical products having similar rts and MS/MS spectra were formed in the chemical and enzymatic synthesis of 5-HT-S and 5-HIAA-S (rt 9.64 min). The rts and MS/MS spectra of chemically synthesized DOPAC-Ss and HVA-S

differed from those that were enzymatically synthesized, indicating that the structures of the products are different. The yield of sulfates in the chemical synthesis was sufficient for NMR studies (over 2 mg). The chemical shifts of the H4 and H6 protons of 5-HT-S and 5-HIAA-S (rt 9.64 min) were clearly moved downfield, compared with those of free substrates, indicating that sulfation occurred at the phenolic group. NMR studies of chemically synthesized DOPAC-Ss and HVA-S showed that only two aromatic hydrogens were seen in the spectrum, suggesting that sulfation had occurred in the benzene ring. However, similar chemical synthesis has been successfully used for the production of DA-3- and 4-O-Ss [108].

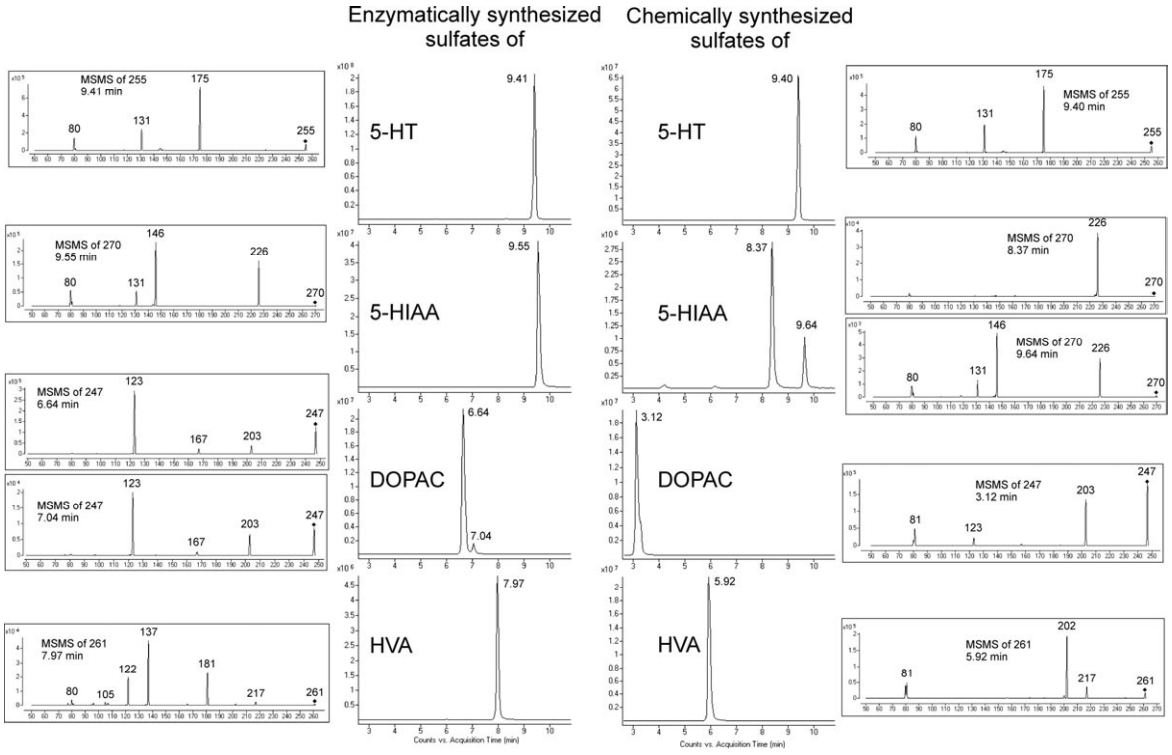

Figure 13. *LC-ESI-MS/MS analysis of enzymatically and chemically synthesized 5-HT-, 5-HIAA-DOPAC- and HVA-sulfates. The negative ion MS/MS spectrum for each chromatographic peak is shown (IV). Linear gradient 5% ACN:MeOH (1:1) for 1 min and 5–20% ACN:MeOH (1:1) for 1-11 min was used.*

In the analysis of brain microdialysates chemically synthesized 5-HT-S and 5-HIAA-S (rt 9.6 min) and enzymatically synthesized DOPAC-Ss and HVA-S were used as standards. The production of HVA-S and DOPAC-Ss on the milligramscale with enzymatic synthesis was not successful and therefore the concentrations of solutions containing HVA-S and DOPAC-S were calculated by determining the concentration of free HVA and DOPAC by LC-MS/MS after acid hydrolysis.

4.3.4 Rat brain microdialysates

The retention of biogenic amine neurotransmitters and their phase I and II metabolites in the reversed-phase chromatography is minimal. Use of the more polar PFP stationary phase provided adequate retention and also the separation of different regioisomers of intact glucuronide and sulfate conjugates (Figs. 12–15). The inorganic salts of Ringer's solution were eluted well before the analytes and were washed to waste. The LODs (S/N = 3) for polar biogenic amines and their glucuronides analysed in the positive ion mode varied between 0.1 and 0.3 nM (10–30 fmol). Intact 5-HT-G and DA-G have not previously been analysed by LC-MS/MS, but the LODs for DA and 5-HT determined by LC-ESI-MS/MS have been reported to vary between 0.2 and 10 nM (2–25 fmol injected into the column) [93, 106]. In a recent study, lower LODs (30 pM, 0.6 fmol) for DA and 5-HT by UPLC®-MS/MS was achieved after derivatization [99]. The LODs (0.5-20 nM, 50–2000 fmol) were higher for negatively charged HVA, DOPAC, 5-HIAA and their sulfate conjugates, but the sensitivity was adequate for the measurement of brain microdialysates. Similar LODs for HVA and DOPAC were reported in a previous study, using LC-MS/MS [126]. The calibration curve for each compound was measured (Tables 1 and 3, III and IV, respectively), and the linearity was good, r^2 being > 0.996 and deviation less than 15%, except in few data points <29%. The intra-assay precision of the method was studied at two concentration levels: 10 and 100 nM. The %RSD values were typically below 4%, indicating good repeatability of the analysis. The matrix effect was studied by spiking brain microdialysates with 2.7 nM DA and DA-G, and 91 nM HVA and DOPAC (III). The recoveries were <111% for DA and HVA, which is acceptable for a bioanalytical method, while the recovery was 119% for DA-G and 81 % for DOPAC. The effect of matrix on ionization of 5-HT, 5-HT-G, 5-HIAA, 5-HIAA-S, HVA-S and DOPAC-S was not studied, and therefore possible suppression effects may distort the quantification of these compounds. Decomposition of DOPAC in brain microdialysates was observed. Six injections were made from a pool of microdialysates collected from the striatum of mouse brains and the concentration of DOPAC decreased from 290 nM to 90 nM during the 4 h storage of the brain microdialysis sample at room temperature, resulting in poor repeatability (%RSD = 37%). However, the concentration of DOPAC (500 nM) diluted in Ringer's solution (standard sample), stored at room temperature and analysed at 1, 13, 26, 36 and 51 h after sample preparation, remained constant (%RSD 2.5%). These results indicate that microdialysate samples should be analysed or frozen immediately after collection to prevent this decrease in concentration of DOPAC.

For the quantitative analysis of rat brain microdialysates the injection volume needed to be high (100 µl) and therefore the rapid changes in neurotransmitter levels in the brain could not be measured (III, IV). However, the LC-MS/MS method was well suited for measuring the levels of glucuronides and sulfates of neurotransmitters and their phase I metabolites, as well as the unconjugated analytes in the brain microdialysates. The compounds were detected by comparing the rts and the intensity ratios of at least two SRM pairs of each compound in brain microdialysates with those of the standards diluted in Ringer's solution. The variation in the intensity ratio of the product ions monitored was

less than 15% and the variation in the relative rts was less than 1.3% in all positive identifications. The SRM chromatograms of the blank Ringer's solution, run before every microdialysis sample, showed no memory effects.

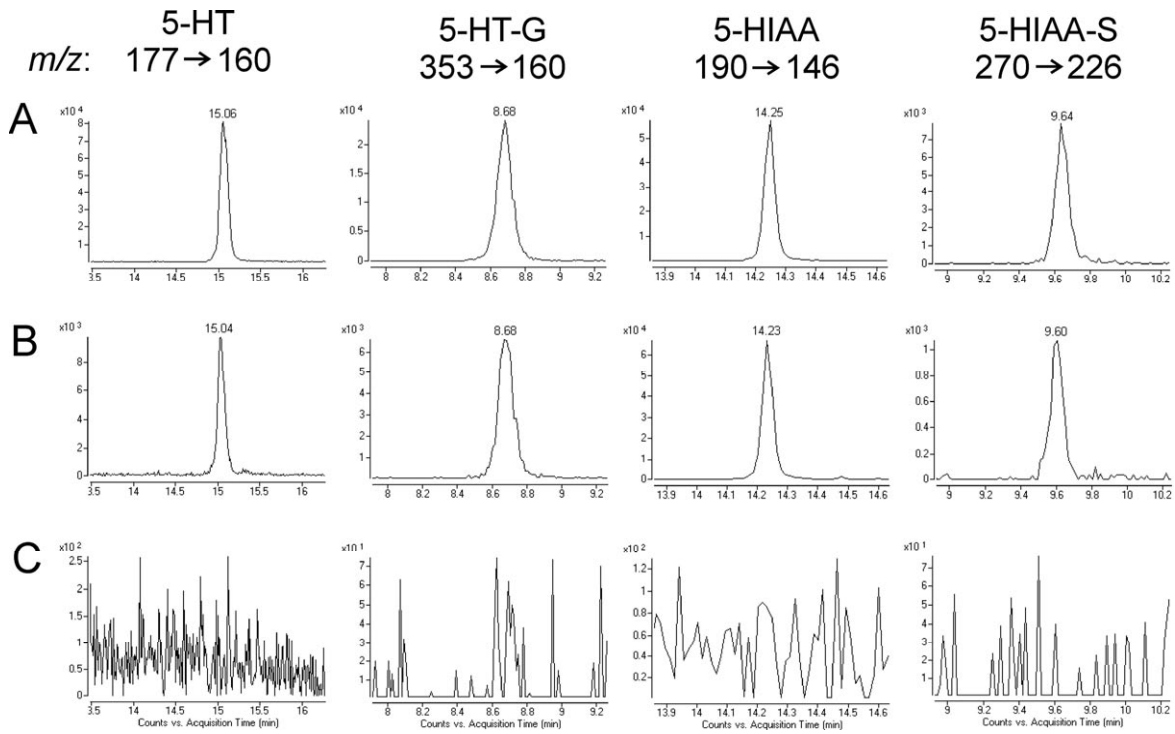

Figure 14. *SRM chromatograms of (A) 5 nM 5-HT and 5-HT-Glu, and 100 nM 5-HIAA and 5-HIAA-sulfate standards diluted in Ringer's solution, (B) rat brain microdialysate and (C) Ringer's solution (blank) (IV). The chromatographic conditions are shown in Table 2.*

For the first time, the intact glucuronide conjugates of DA and 5-HT were unambiguously detected in rat brain microdialysates (Figs. 14 and 15) (III, IV). The concentration of 5-HT-G (1.0–1.7 nM) was over two times higher than the concentration of 5-HT (0.4–2.1 nM) in three out of four rat brain microdialysates analysed (Table 4). For DA the concentration of glucuronide conjugate (1.0–1.4 nM) was similar or lower than the concentration of free neurotransmitter (1.2–2.4 nM). In previous studies an acid-hydrolysable conjugate of 5-HT was detected in superfusates of rat spinal cord [80], but the glucuronidation of 5-HT has not been shown. 5-HT is a good substrate for UGT 1A6 [20], which is expressed in human [20] and rat brain [19], supporting the presence of 5-HT-G in rat brain microdialysates found in the present study (IV). DA-G was found in rat [22] and human [23] CSF, which is assumed to reflect the metabolism of neurotransmitters in the brain. The glucuronides of 5-HIAA, DOPAC or HVA in rat brain microdialysates were not detected in our study (IV).

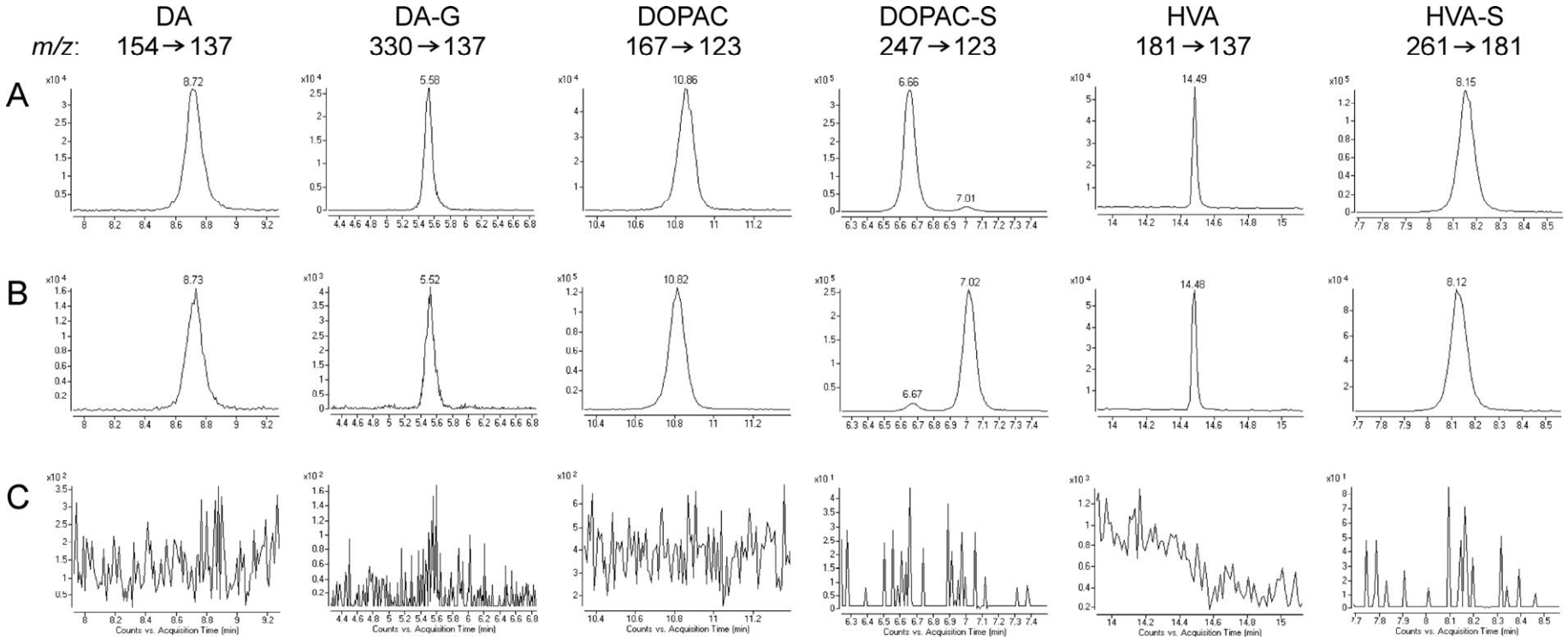

Figure 15. *SRM chromatograms of (A) 5 nM DA and DA-Glu, 300 nM DOPAC and HVA, 360 nM DOPAC-sulfate and 100 nM HVA-sulfate standards diluted in Ringer's solution, (B) rat brain microdialysate, and (C) Ringer's solution (blank) (IV). The chromatographic conditions are shown in Table 2.*

Table 4. *Analytical results of rat brain microdialysates by LC-MS/MS.*

	Concentration, nM			
Compound	Animal 1	Animal 2	Animal 3	Animal 4
5-HT	0.4*	0.5	0.7	2.1
5-HT-G	1.0	1.3	1.7	1.5
5-HIAA	110	140	120	150
5-HIAA-S	7*	11*	12*	13*
DA	1.2	2.0	2.4	2.1
DA-G	1.1	1.4	1.0	1.0
DOPAC	890	910	700	980
DOPAC-S (rt = 6.6 min)	40	40	40	40
DOPAC-S (rt = 7.0 min)	280	290	280	400
HVA	510	510	360	540
HVA-S	90	90	80	100

* < LOQ, only an estimation

The rat brain microdialysate analysed with LC-MS/MS contained 5-HIAA-S, HVA-S and two DOPAC-S regioisomers, most probably DOPAC-3- and -4-O-S (Fig. 14). The sulfation of DOPAC, HVA and 5-HIAA in rat brain has also been observed in previous studies, where a clear increase in the concentration of free substrate after enzymatic sulfatase hydrolysis was observed [27, 28]. We observed that app. 10%, 15% and 30% of 5-HIAA, HVA and DOPAC, respectively, was in sulfated form in rat brain striatum microdialysates (Table 4). In other studies the conjugated phase I metabolites were extracted from brain samples, and the amounts of conjugated 5-HIAA, HVA and DOPAC after acid hydrolysis were 10%, 20–36% and 12–32%, respectively, of the total amount (concentration of conjugated and free substrate) in the rat brain striatum [78, 79, 127]. These concentration ratios are quite similar to those obtained in our study in microdialysates collected from the rat brain striatum (IV). Neither DA-S (LOD 0.8 nM, 80 fmol) nor 5-HT-S (LOD 0.1 nM, 10 fmol) was detected in rat brain microdialysates (III, IV), even though DA-S was detected in rat CSF [22] and brain samples [26] after sulfatase hydrolysis. In a previous study the sulfate conjugates of 5-HT and DA were detected in human CSF [23].

5. SUMMARY AND CONCLUSIONS

The concentration of neurotransmitters and their metabolites in brain microdialysates is low (fmol amounts) and the sample volume is limited (typically < 40 µl). The capability for analysing small sample volumes is desired so that the chemical changes in the brain can be monitored over short time intervals. The perfusion fluid used in the microdialysis contains a high concentration of inorganic salts (150 mM), whereas the concentration of small polar neurotransmitters is low, making demands on chromatographic separation. Many compounds having similar structures are coextracted from the brain, which complicates method development even with specific LC-MS/MS. Low LODs and good specificity are required of the analytical method to be able to analyse low concentrations of neurotransmitters accurately and therefore triple-quadrupole instruments and SRM mode was selected for the detection in this study. LC-MS/MS methods were developed for the analysis of neurotransmitters and their phase I and phase II metabolites in the brain microdialysates. Another aim of this study was to identify glucuronide and sulfate conjugates of neurotransmitters in the brain. However, fully validation of the methods was not performed and in future applications it should be done to ensure the reliability of quantitative results.

The sensitivity of ESI-MS analysis is not optimal for small polar compounds. In the present study the ionization efficiency of polar amino acids was improved by converting the polar nature of amino acids to a more hydrophobic by derivatization. Retention in reversed-phase chromatography was increased and the amino acid derivatives were eluted at higher organic contents, which also enhanced ESI sensitivity. The LODs for amino acids derivatized with FMOC, PrCF or butanol were 2–60 times lower than for native underivatized amino acids. The separation of amino acids from the suppressive inorganic ions of Ringer's solution was also achieved by FMOC and PrCF derivatization. The analysis of GABA in rat brain microdialysates by LC-MS/MS has not been presented previously, due to high LODs. After the PrCF derivatization used in this study, GABA could be easily quantified in rat brain microdialysates by LC-MS/MS. The separation of GABA from its isomers α-and β-ABA, also extracted from the brain, was achieved. The drawback of the LC-MS/MS method developed was the memory effect observed for some amino acids, complicating analysis at low concentrations.

The separation of polar compounds in reversed-phase chromatography requires either derivatization of the analyte or ion-pair additives in the eluent. However, the derivatization increases sample pretreatment and ion-pair reagents cause signal suppression in ESI. A good alternative is the use of polar stationary phases in which the polar analytes have higher affinity. In HILIC the compounds are eluted at high organic content, which increases the ESI sensitivity. Low LODs were achieved for Ach and Ch in present study, using HILIC-LC-MS/MS. The concentration of Ach in rat and mouse brain microdialysates, without the use of acetylcholinesterase inhibitors, was clearly above the LOQ with the LC-MS/MS method developed. Those compounds having the same molecular weight and collision induced dissociation as Ach and the ISTD, were

chromatographically separated. This was the first time that HILIC was employed for the analysis of Ach in brain microdialysates and thereafter it has also been exploited in other publications discussing the analysis of brain microdialysate by LC-MS/MS. The clogging of the MS interface observed in this study was reduced by changing the stainless-steel union of the ion-source to a more inert alternative but clogging might still be a problem in routine analysis. Therefore the method should be modified so that the entry of chloride ions to MS is prevented.

So far indirect methods, employing either acid or enzymatic hydrolysis, have been used in the analysis of sulfate and glucuronide conjugates in the brain. Indirect methods are prone to errors, due to the hydrolysis step, and the presence of conjugate is difficult to confirm, especially if the concentration of conjugate is low compared with that of free aglycon. Even though the type of conjugate can be studied with more specific enzymatic hydrolysis, the conjugation site cannot be identified. In the present study, direct quantitative LC-MS/MS methods for the analysis of intact sulfate and glucuronide conjugates in brain microdialysates were developed. With a direct method even low concentrations of sulfate and glucuronide conjugates were measured and the different regioisomers were also separated. Using MS/MS detection, estimations of the glucuronidation site (aliphatic versus phenol-linked glucuronides) could be presented. A large injection volume (100 µl) was needed to be able to quantify 5-HT-G and DA-G in rat brain microdialysates. However, the quantification of analytes in smaller sample volumes is required for the follow-up of rapid changes in the concentration levels of the compounds in the brain. One strategy for the development of a more sensitive method for the analysis 5-HT, DA and their metabolites may be the use of miniaturized LC-MS/MS systems

In our study the glucuronidation of 5-HT and DA in rat brain was shown for the first time. The results suggested that neurotransmitters are glucuronidated and their phase I metabolites are rather sulfated. Further studies are needed to determine the meaning of this metabolism in the brain. What happens to the conjugates? Do they accumulate in the brain or do they use transporters? Can glucuronides be hydrolysed back to active neurotransmitters? What specific enzymes are expressed in the brain and produce these sulfate- and glucuronide-conjugates. Pharmacological experiments are needed to determine whether the concentrations of phase II metabolites are altered in different diseases compared with normal basal levels and what effect different drugs have on phase II metabolites. What happens if the phase II metabolism pathway is blocked in the brain? It is known that metabolic pathways may differ greatly between species and therefore it is highly important to explore what occurs in the human brain. SULT 1A3, found in human but not in rat brain, has high affinity for DA. Does the glucuronidation of DA still occur in human brain or is the sulfation more favourable phase II metabolism reaction?

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