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Review

Amino acid neurotransmitters: separation approaches and diagnostic value

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Abstract

Amino acids in the central nervous system can be divided into non-neurotransmitter or neurotransmitter depending on their function. The measurement of these small molecules in brain tissue and extracellular fluid has been used to develop effective treatment strategies for neuropsychiatric and neurodegenerative diseases and for the diagnosis of such pathologies. Here we describe the separation and detection techniques that have been used for the measurement of amino acids at trace levels in brain tissue and dialysates. An overview of the function of amino acid transmitters in the brain is given. In addition, the type of sampling techniques that are used for the determination of amino acid levels in the brain is described.

Keywords: Reviews; Amino acid neurotransmitters

Contents

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1. Introduction—amino acids in the central nervous system and their function

Over the last 50 years a growing body of evidence has been accumulating to indicate that the most widely distributed neurotransmitters belong to a class of compounds referred to as amino acids. These small molecules are present at high concentrations in all cells probably because of their key role in protein synthesis and metabolism.

Glutamate and aspartate are the most abundant free amino acids in the mammalian brain and are classed as excitatory neurotransmitters [1,2] that are released in a Ca^{2+}-dependent manner. Their excitatory effect is variable, but has been shown to be dependent on their relative agonist potency [3–5] at ionotropic or metabotropic receptor subtypes. In addition to its role as a neurotransmitter in the CNS, glutamate has an array of functions associated with metabolic regulation. Thus, glutamate that is involved in neurotransmission is differentiated in the brain from the pool that is involved in various metabolic functions by complex compartmentation. The concept of multiple glutamate pools in the brain [6] suggests that at least two such metabolic compartments can be defined based on the proportion of total glutamate pool that is ascribed to each compartment. The so-called small compartment is thought to correspond to glutamate metabolism occurring in astrocytes, whereas the large compartment would correspond to metabolic activity taking place in nerve cells. In the brain, glutamate is used by a rather important number of descending pathways originating from neocortical pyramidal cells, intrahippocampal and hippocampal projections and parallel fibres of the cerebellar cortex [6,7]. There is also considerable evidence that glutamate is the transmitter of choice in excitatory interneurons in the spinal cord as well as in terminals of primary afferent neurons [8]. Hence, the implication of glutamate in neurological disorders (cerebral ischemia, hypoxia and epilepsy) as well as mechanisms of synaptic plasticity, learning and memory. Furthermore, recent findings support the idea of a hypoactive amino acid system contributing to the aetiology of schizophrenia. In fact, decreased concentrations of glutamate and aspartate have been reported in the prefrontal cortex and hippocampus of schizophrenic patients [9]. In addition, the activity of N-acetylated alpha-linked acidic dipeptidase (NAALADase), which cleaves *N*-acetylaspartyl glutamate (NAAG) to *N*-acetyl aspartate (NAA) and glutamate, seems to be lower in both the anterior cingulate cortex and hippocampus of schizophrenics [10,11]. The case for aspartate as a neurotransmitter has received less attention, but a few studies seem to suggest that aspartate is acting as a transmitter in specific cortical and hippocampal pathways [12,13], in climbing fibres originating from the inferior olive of the medulla and making synapses onto cerebellar Purkinje cells [14] and in some primary afferent neurons [15]. Given that aspartate has a higher affinity for the transporter than glutamate, but a lower affinity for the glutamate receptor, it may well be the case that aspartate levels serve as an index for reverse transport without having further physiological effects. This issue also deserves further investigations.

γ-Aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the CNS. In fact, as many as 10–40% of nerve terminals in the cerebral cortex, hippocampus, and substantia nigra may use GABA as a neurotransmitter [16]. Furthermore, GABA is also expressed in the cerebellum, striatum, globus pallidus and olfactory bulbs. Finally, GABA plays a rather important role in the spinal cord as revealed by its presence throughout the spinal gray matter except for motor regions [7]. Two main types of GABAergic receptors have been identified and these are usually referred to as $GABA_A$ and $GABA_B$. $GABA_A$ receptors are ligand-gated channels and blockade of these receptors can lead to powerful convulsant effects. However, a number of sedative–hypnotic drugs can also positively modulate the influence of GABA on the $GABA_A$ receptor. Thus, the $GABA_A$ receptor is a glycoprotein made up of at least five different subunits. It seems that cells can assemble certain combination of subunits more readily than others can, which may explain the locoregional heterogeneity of $GABA_A$ receptors and their differential responsiveness to benzodiazepines and ethanol. The $GABA_B$ receptor is a G-protein-coupled receptor that is present both postsynaptically and on nerve terminals where it can inhibit neurotransmitter release. The $GABA_B$ receptor can act via at least four different effector mechanisms including inhibition of adenylyl cyclase, stimulation of phospholip-

ase A_2, activation of potassium channels and inhibition of calcium channels. Altogether, these findings suggest that GABA is a key transmitter for inhibitory control mechanisms in the brain. Hence, the main implication of GABA is in the pathophysiology of epilepsy and anxiety, but also schizophrenia.

Glycine is the second major inhibitory neurotransmitter in the CNS. Its role as a neurotransmitter has been most conclusively established in inhibitory interneurons in the spinal cord. Evidence for the involvement of glycine in brain pathways has also been obtained as reflected by its presence in the corticohypothalamic tract, spinal afferents from the raphe nuclei and reticular formation and in interneurons located in the striatum and substantia nigra [7,17]. The glycine receptor functions in a similar way as the $GABA_A$ receptor. It contains a Cl^- channel and glycine-mediated neuronal hyperpolarization is caused by increased Cl^- conductance across the membrane. The inhibitory effects of glycine are blocked by strychnine that has been shown to have potent convulsant effects. The localization of glycine receptors and the effects of strychnine intoxication suggest that glycinergic systems play an important role in sensorimotor function and abnormal startle responses. Hence, the involvement of glycine in the pathophysiology of schizophrenia.

Although histamine is not an amino acid it is usually presented in the amino acid class of transmitters. Histamine is synthesized from the decarboxylation of the amino acid histidine and is best known for its intimate relationship with respiratory allergies to airborne pollens. Histaminergic cell bodies exhibit a rather restricted localization in the CNS where they can be found exclusively in the tuberomammillary nucleus in the basal posterior region of the hypothalamus [18]. However, cell bodies give rise to widespread ascending projections and immunohistochemical studies indicate that neuronal histamine is co-localized with GABA, adenosine deaminase (a suggested marker for cells that are using adenosine as a transmitter) as well as several neuropeptides such as galanin [18]. Histamine has been involved in a considerable amount of functions including ingestive behaviours, sleeping and arousal, motor activity, sexual behaviour, aggression, pain, pituitary gland activity, thermoregulation and regulation of blood pressure [18]. Thus, the diversity and ubiquity of these effects may indicate a rather widespread and basic role in neural functioning. Furthermore, the configuration of varicose fibres elaborated by histaminergic axons together with the morphological organisation of the histaminergic system as a small number of cell clusters with diffuse efferent projection pathways suggest that histamine may act at a distance from its release site, perhaps in a neuromodulatory manner. Finally, the sulphur-containing amino acids cysteine sulphinate (CSA), homocysteine sulphinate (HCSA), cysteate (CyA) and homocysteate (HCA) that are generated in the trans-sulphuration pathway are excitatory in nature and are thought to play a neurotransmitter role in one or more pathways within the mammalian CNS [19,20].

Altogether these findings demonstrate that amino acid neurotransmitters represent a major class of biochemicals that are involved in neuronal communication at synapses in the CNS. Hence, analysis of these molecules in neuropsychiatric diseases may provide a means of diagnosis of disease and possible treatment strategies. The methods that have been reported for the measurement of amino acids can be divided into two categories: biochemical assays that involve the determination of a single amino acid and chromatographic procedures. The separation techniques that have been used for resolving neurotransmitter amino acids include TLC [21], achiral HPLC with electrochemical or fluorescence detection, chiral HPLC, CE, GC–MS and LC–MS.

2. Sampling techniques and sample preparation

2.1. Brain tissue

A number of different approaches have been reported for the preparation of brain tissue samples for the measurement of neurotransmitter amino acids. The first step is common and involves the separation of soluble proteins and peptides from free amino acids. Deproteination of brain samples can be carried out by homogenising the tissue in acid [22] or acidified solvent such as methanol [23] or a cold solvent [24] and separating the insoluble matter by centrifugation. Using aqueous strong acid in this step

can lead to hydrolysis of asparagine and glutamine to aspartate and glutamate, respectively. Hence, to minimise hydrolytic conversion, dilute acid in an organic solvent is used. The supernatant is then treated with a derivatising agent [25] or analytes can be removed from the supernatant using solid-phase or liquid–liquid extraction prior to derivatisation [26]. Although the measurement of neurotransmitter amino acids in brain tissue samples is potentially useful, the interpretation of the data can be difficult. This arises primarily because amino acids like glutamate serve a metabolic and a neurotransmitter function in the brain. Hence, it is not feasible to distinguish between glutamate associated with general metabolism from that related to transmitter function. However, this does not hold for all neurotransmitter amino acids because tissue levels of GABA that serve a transmitter role have been shown to represent between 70 and 80% of the total pool [27].

2.2. Microdialysis

Microdialysis is a technique that involves continuous sampling of the extracellular fluid of living organisms by way of a hollow fibre dialysis membrane [28]. Commercially available dialysis membranes are 0.2–0.3 mm in diameter; these are attached to a cannula which has inlet and outlet tubes. The cannula is surgically implanted into the tissue to be studied and perfused with an iso-osmolar physiological fluid. As a result of the concentration gradient between the perfusate and extracellular space, small molecules traverse into the fluid. The outflow is either collected at fixed time intervals or loaded directly onto a chromatographic system. The sample volume required dictates the time interval, and each sample represents an average concentration value obtained over this time period. The recovery of analytes is dependent on the flow-rate of the perfusate, the dimensions and properties of the dialysis membrane, the molecular mass and hydrophobicity of the molecule. More recently the technique has been used in clinical neuroscience settings [29]. The level of amino acids in dialysates in 15 different regions of the rat brain has been shown to be between 0.7 and 151 pmol μl^{-1} [24].

Several authors have questioned the vesicular origin of amino acids such as GABA and glutamate as detected in the extracellular fluid by microdialysis [30–34]. In fact, a major part of extracellular GABA and glutamate does not seem to fulfil unequivocally the classical criteria for exocytotic release as measured by dependence on opening of sodium channels by using tetrodotoxin (TTX) and availability of Ca^{2+} under both basal and stimulated conditions. However, the well-established sensitivity to TTX and Ca^{2+} for neurotransmitters such as monoamines and acetylcholine can be interpreted in terms of impulse flow dependence because these transmitters are present in a single pool in which they are taken up after neuronal discharge. This, however, is not the case of GABA and glutamate that do not fulfil such conditions. Thus, extreme care should be taken when interpreting extracellular levels of amino acids as directly related to neurotransmission. The very fact that both glutamate and GABA are present in different pools in the brain suggests that changes in extracellular levels of these amino acids may originate from either changes in exocytotic release, carrier-mediated release or leakage of glial cells as a result of changes in pH, ion concentrations of the extracellular fluid or local opening of the blood–brain barrier. Thus, compartmentation of amino acid transmitters is also probably reflected in their extracellular content that represents a mixture of both metabolic and synaptic pools. The extent to which the metabolic pool is related to its synaptic counterpart is currently unknown and would warrant further investigations.

2.3. Voltammetry

Voltammetry is a polarographic methodology for direct in situ and real time measurement of electronically active chemicals without the need for sample preparation or chromatographic analysis. Different types of voltammetry are available, but techniques typically used in biological studies are chronoamperometry, linear voltammetry, cyclic voltammetry and pulse voltammetry. Voltammetry is based on the application of a ‘‘dynamic’’ oxidation (or Ox–Red) potential and the analysis of the electrons ‘‘freed’’ by the compound(s) under measurement (only chronoamperometry and related methods such as direct current amperometry, apply a specific, fixed

Ox potential). Less popular methodologies are ''stripping voltammetry'' or ''sinusoidal voltammetry''.

Voltammetry is applied via a three-electrode potentiostat system as described previously [35]. Briefly, it consists in a reference, an auxiliary and a working electrode. The reference and the auxiliary are usually a silver/silver chloride and silver wire, respectively. For in vivo studies these two electrodes are ~100 μm in diameter. The working electrode is mainly either a metallic or carbon-based sensor. The former is gold, silver or platinum based, whereas the latter (mostly used in biological studies) is mainly carbon paste or carbon fibre electrodes.

Native amino acids can be detected at a copper microelectrode using sinusoidal voltammetry [36], and most of the underivatized amino acids that are electro-inactive under conventional amperometric (voltammetric) conditions can react rapidly with the electrogenerated bromine [37]. On the other hand, the oxidation potential of derivatized amino acids could be monitored via cyclic voltammetry [38]. Cysteine and methionine were detected on a platinum electrode via linear sweep voltammetry [39], while nickel titanium alloy electrodes exhibit high stability for constant-potential amperometric detection of amino acids in flow systems, i.e. arginine, leucine and isoleucine are measured with detection limits ranging from 0.9 pmol for the former to 90.2 pmol for the other two [40]. Nickel–copper alloy electrodes coupled with chronoamperometry are used to detect cysteine and glycine [41], while cyclic voltammetry performed within an artificial perylimph solution (similar to mammalian cochlea perylimph which contains amino acids and proteins) shows that many amino acids, and in particular the sulphur-containing cysteine and methionine are adsorbed at the platinum electrode [42].

The electrochemical oxidation of cysteine, tyrosine and tryptophan has been demonstrated using differential pulse voltammetry associated with carbon fibre electrodes [43]. Similarly, the electrochemical oxidation of cysteine, tyrosine and tryptophan at graphite–methacrylate composite electrodes associated with cyclic voltammetry shows that these amino acids present characteristic oxidation waves [44] that can be registered between 400 and 925 mV. It has been suggested that the signal occurring at ~900 mV is related to the specific oxidation wave of tryptophan [45]. Cysteine, cystine and glutathione give a cathodic stripping voltammetric peak in the presence of nickel ion, due to the catalytic reduction of the ion [46]. Conducting electroactive polymer modified electrodes have also been applied to detect amino acids. They were responsive to four amino acids: aspartic acid, serine, alanine and arginine, with maximum response obtained for the former [47]. An application of these studies is the in vitro analysis of amino acids in biological fluids [48,49]. The utilisation of catechol as an electrochemical indicator for the presence of cysteine, homocysteine and glutathione, i.e. amino acids possessing sulphydryl thiol functions has also been described [50]. The electrogeneration of *o*-quinone was followed by a 1,4-addition reaction with available cysteine (or/and homocysteine and glutathione). The increase of current, due to the re-oxidation of the thiol–catechol adduct, would allow the quantification of the concentration of the thiol-related amino acids. Finally, another original method lies in the combination of open tubular liquid chromatography and voltammetry in order to determine trace levels (femtomoles) of tyrosine and tryptophan within individual neurons [51]

In vivo studies using mainly linear sweep voltammetry or differential pulse voltammetry propose a high sensitivity of carbon-based electrodes for ascorbic acid or vitamin C [52–55]. These authors claim that the extracellular levels of vitamin C reflect the release of excitatory amino acids in the brain. Similarly, recent in vivo data have shown that direct current amperometry and differential pulse voltammetry can detect selectively the oxidation of nitrogen monoxide (NO) ex vivo or in vivo, i.e. within the endothelium of rat aortic rings or in discrete rat brain areas, respectively [56,57].

3. Chromatographic assays for the measurement of neurotransmitter amino acids

3.1. HPLC

3.1.1. Background

The majority of amino acids are small aliphatic molecules that do not possess either fluorescent or

strong UV–Vis absorbance characteristics. Thus, HPLC analysis with UV–Vis or fluorescence detection cannot be used for the analysis of these molecules. In simple solutions they can be directly measured at pmol levels using HPLC with evaporative light-scattering detection [58]. However, this technique is unsuitable for the measurement of amino acids at trace levels in biological matrices. To improve both the selectivity and sensitivity of detection of amino acids in biological samples they are derivatised. The majority of methods that have been reported for determination of amino acids are based on precolumn derivatisation with an amine reagent.

3.1.2. HPLC with fluorescence or electrochemical detection

Precolumn derivatising reagents that have been used for the analysis of transmitter amino acids include 1-dimethyl aminonaphthalenesulfonyl chloride (DANSYL) [59], phenylisothiocyanate (PITC) [60], 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) [61], *ortho*-phthalaldehyde (OPA) [62] and naphthalene-2,3-dicarboxaldehyde (NDA) [22]. Although PITC and DANSYL form stable derivatives they react with water to generate hydrolysis products that are observed as intense HPLC-peaks in chromatograms. Also, for assays that are based on using PITC the excess reagent has to be removed prior to analysis, adding a step to the procedure. *Ortho*-phthalaldehyde is a primary amine reagent that has been used extensively for the analysis of transmitter amino acids at trace levels. The primary advantage of using OPA over other reagents is that it itself is not fluorescent [63,64] or electroactive [65,66] whereas the derivatives have suitable fluorescent and electrochemical characteristics. The reaction of OPA with amino acids can be carried out in the presence of an alkyl thiol [67] to give 2,2-disubstituted isoindole derivatives. The stability of OPA derivatives formed using alkyl thiols is poor [68]. In an effort to increase the stability Jacobs [69] used sulphite ions to form *N*-alkyl-isoindole-sulphonate derivatives that were fluorescent, electroactive and more stable. This assay has been used successfully for the measurement of neurotransmitter amino acids [69]. However, sulphite is electroactive and it may interfere with the HPLC analysis of amino acid derivatives assays based on electrochemical detection. Also, the isoindole sulphonate derivatives are susceptible to hydrolysis at pH below 5 [68]. To minimise baseline drift and increase throughput, isocratic separation conditions are preferred for HPLC analysis based on ECD. However, gradient elution with ECD has been reported for the analysis of transmitter amino acids in dialysates derivatised with OPA [70,71]. *Ortho*-phthalaldehyde has also been used for the analysis of excitatory sulphur-containing amino acids, CSA, HCSA, CyA and NCA [72]. A further application of OPA for transmitter amino acids is measurement of D- and L-amino acid in brain tissue [26]. The reagent reacts with amino acids in the presence of the *N*-acetyl-L-cysteine to give diastereoisomers that can be resolved using reversed-phase chromatography.

To circumvent the problems associated with precolumn assays based on using OPA, naphthalene-2,3-dicarboxaldehyde (NDA) was designed and developed by de Montigney et al. [73]. This reagent reacts with primary amines in the presence of cyanide to produce 1-cyanobenz[*f*]isoindole (CBI) derivatives. The derivatives formed with amino acids are significantly more stable than the corresponding OPA counterparts and exhibit a higher quantum efficiency [73]. As with many OPA amino acid derivatives, the CBI derivatives are fluorescent [73,74] and electroactive [75,76]. The reagent has been used extensively in our laboratories for the measurement of amino acids in brain tissue and dialysates using fluorescence detection [22,77]. Fig. 1 shows a chromatogram of amino acid standards in buffer and dialysate samples treated with NDA. The analysis time per sample was found to be too long to allow the large number of samples to be analysed within a suitable time. As a result a more rapid HPLC assay was developed using a monolithic C_{18} column. Fig. 2 shows a chromatogram of a mixture of 17 CBI-amino acids in buffer resolved within 10 min. This assay has been used successfully for the measurement of transmitter amino acids in microdialysis samples from subregions of the rat nucleus accumbens [77].

3.2. CZE with laser-induced fluorescence detection

The temporal resolution that is achieved in microdialysis is dependent on the sample volume that is

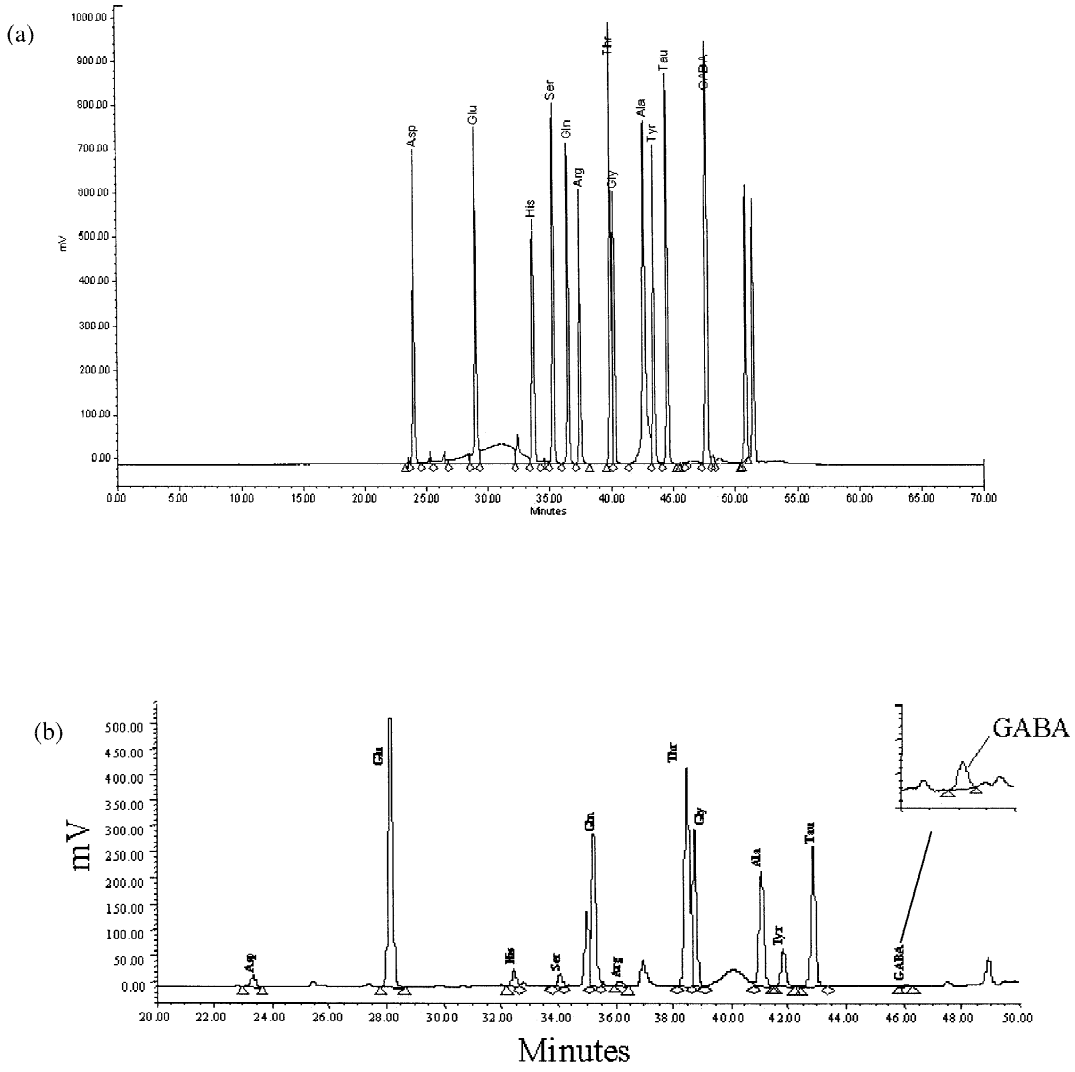

Fig. 1. Chromatograms of (a) mixture of amino acid standards each at a concentration of 2 μ*M* and (b) microdialysates from nucleus accumbens of rat brain derivatised with NDA-CN. Samples (16 μl) were treated sequentially with 0.1 *M* borate buffer (pH 9.2) (16 μl), 10 m*M* aqueous sodium cyanide (2 μl) and 3 m*M* NDA (16 μl). The samples were left to incubate at room temperature for 5 min. An aliquot (5 μl) was loaded onto a 100×2.1 mm I.D. X-Terra MS C_{18}, 3.5 μm column. The separation was carried out using a binary gradient elution profile comprising of eluent ''A'' 50 m*M* ammonium acetate (pH 6.0)–THF (95:5, v/v) and eluent ''B'' 50 m*M* ammonium acetate–acetontirile–methanol (35:55:10, v/v). A gradient from 10 to 41% B over 25 min was used. The flow-rate was set at 0.31 ml min^{-1}. The column was thermostated to 55 °C. Eluates were detected using a fluorescence detector with excitation and emission wavelengths set at 442 and 480 nm, respectively. The gain and response time were set to 1000 and fast, respectively.

required for analysis. To minimise the sample volume required, and thus improve the time resolution, capillary electrophoresis with laser-induced fluorescence detection (CE–LIF) has been used as an alternative to other analytical techniques. This technique offers high sensitivity, high separation efficiency and rapid separation for a range of neurotransmitters including transmitter amino acids [78–80]. The major issue with the analysis of dialysates using CE is that the small sample volumes are difficult to collect and inject. This problem is compounded by the loss of sample due to evapora-

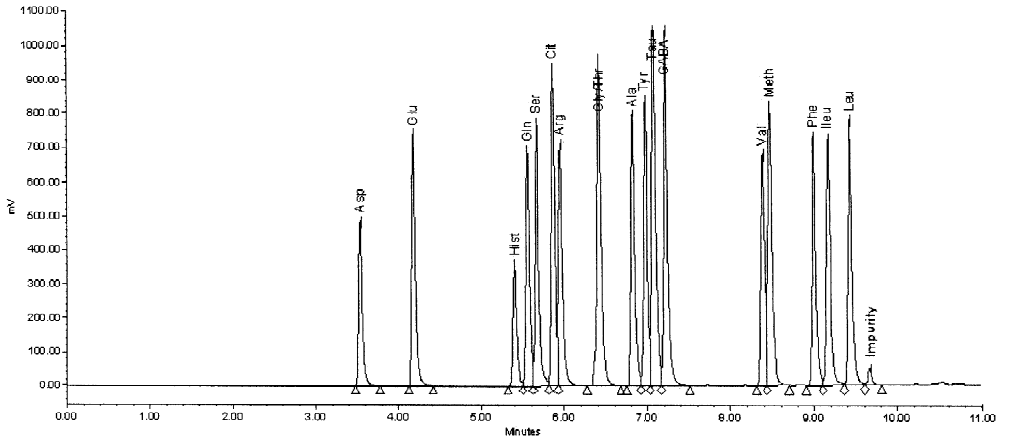

Fig. 2. Chromatogram of a mixture of amino acids each at a concentration of 5 μ*M* in buffer treated with NDA-CN. The sample was derivatised using the procedure described for Fig. 1. An aliquot (5 μl) of derivatised sample was loaded onto a 100×4.6 mm I.D. Chromolith SpeedRod RP 18e column connected in series to a 50×4.6 mm I.D. column containing the same stationary phase. The separation was carried out using a ternary gradient system consisting of ''A'' 50 m*M* ammonium acetate (pH 6.8), ''B'' acetonitrile and ''C'' methanol. A gradient from 85:5:10 to 40:40:20, v/v A:B:C over 10 min was used. The separated analytes were monitored using the detection parameters described for Fig. 1.

tion and adhesion to the sample tube during collection and analysis. To address these issues, systems have been designed in which the outlet from the microdialysis probe is coupled via a mixing tee to pumps that add the individual reagents. The derivatised samples are then analysed either off-line or on-line [81,82]. These systems are typically comprised of a microinfusion pump to perfuse the implanted probe with aCSF, a second infusion pump to deliver derivatising reagent(s) and mixing-tees. In addition, on-line systems include a reaction coil, a microinjection valve and a CE system. It is feasible to set up this type of system because microdialysates are protein-free and therefore no protein precipitation step is necessary prior to injection. Although CE–LIF assays give good limits of detection the use of the technique for the measurement of transmitter amino acids in brain tissue has not been reported. Furthermore, the high efficiency that is obtained with CE can be used in the presence of a chiral selector for the measurement of enantiomers of transmitter and non-transmitter amino acids in brain tissue samples.

3.3. Gas chromatography with mass spectrometry

To analyse amino acids using gas chromatography (GC) they have to be converted to more volatile analytes. This usually involves esterification followed by perfluoroacylation. The amino acid is usually esterified using isobutanol or isopropanol or the corresponding perfluorinated alcohol, the resulting ester can then be acylated with either trifluoroacetic anhydride [83] or pentafluoroacetic anhydride [84] or heptafluorobutyryl anhydride [85]. The amino acid derivatives can be monitored using either electron capture or mass spectrometric detection [83]. The limit of detection that is obtained with these two types of detectors is comparable and allows measurement of amino acids in brain perfusates [85]. The advantage of using mass spectrometry over other GC detectors is that the selectivity offered is greater. A further advantage of the mass spectrometric detector is its ability to distinguish between analytes labelled with stable isotopes, thereby allowing them to be used as internal standards. Dueterated amino acids have been used as internal standards for quantitation of transmitter amino acids in brain perfusates using GC and chemical ionisation MS in the negative ion mode [86]. Capillary GC–MS in the electron-impact mode has been used to study the different pools of neurotransmitter amino acids in brain slices [87]. However, several drawbacks are associated with GC–MS assays. One major disadvantage with using GC–MS methods is the time-consuming sample clean-up procedures. In

comparison CE and HPLC–MS procedures for measurement of amino acids are relatively simple and offer similar selectivity.

3.4. CE and HPLC with mass spectrometry

The coupling of CE with MS has been used for the determination of neurotransmitter amino acids in brain samples [88,89]. This technique offers the advantages of rapid analysis and low sample consumption. One drawback with CE is the relatively low concentration sensitivity. High sensitivity is required for the measurement of neurotransmitter amino acids in brain samples. An approach that has been used to overcome this is transient-isotachophoresis [88]. This technique allows a large injection volume to be loaded onto the capillary [90] with little or no effect on the resolution. Using this method, LODs of between 5 and 20 n*M* have been reported for the measurement of glutamate and GABA. An alternative to this approach is HPLC with MS detection. Although the separation efficiency achieved using HPLC is significantly lower it offers higher concentration sensitivity than CE and the instrumental set-up is less complex. Ma et al. [91] reported the use of HPLC with atmospheric chemical ionisation (APCI) mass spectrometry for the simultaneous measurement of glutamate and GABA in six regions of the brain in rats treated with 3-mercaptopropionic acid. This is an inhibitor of glutamate decarboxylase that has been shown to produce seizures in mice [92]. The use of HPLC with atmospheric chemical ionisation in the negative ion electrospray mode has been reported for the confirmation of identity of CBI derivatives of amino acids [22] detected in brain tissue samples from rats (Fig. 3).

4. Biological applications

A range of chromatographic procedures has been used to study changes in neurotransmitter amino acid levels in neuropsychiatric disorders such as Alzheimer's disease (AD), aseptic meningitis, Down's syndrome, Huntingdon's disease (HD), multiple sclerosis (MS), Parkinson's disease (PD) and schizophrenia. The majority of these assays are based on HPLC analysis of ante-mortem, post-mortem, or CSF samples. More recently, in vivo microdialysis has been used as an in situ clinical monitor in neurointensive medicine.

Precolumn derivatisation with OPA–2-mercaptoethanol has been used to study changes in neurotransmitter amino acid levels in post-mortem tissue from those afflicted with AD [93], Down's syndrome [94] and PD [95]. In post-mortem caudate nucleus, nucleus accumbens, frontal cortex, amygdala and hypothalamus of chronic schizophrenics it has been shown that levels of aspartate, GABA, glutamate, glycine and taurine are unchanged with the exception of a decrease in the concentration of GABA in the amygdala [96]. The OPA–mercaptoethanol assay has also been used to investigate changes in levels of neurotransmitter amino acids in HD cerebellar cortex and dentate nucleus [97]. The authors found no significant changes in levels of glutamate, taurine or GABA whereas concentration of aspartate was elevated by 21% in cerebellar cortex. Data recorded on levels of neurotransmitter amino acids in post-mortem tissue have to be interpreted with caution because there are many variables associated with this type of sample. This includes cause of death, the temperature at which the body is kept before sampling, the method of sampling, anatomical precision of dissection, the length of time between sampling and freezing of the tissue and duration of storage. Perry et al. [98] studied post-mortem changes in amino acids and found that glutamate and taurine remain constant in the human brain. However, levels of aspartate, glycine and GABA were found to rise. Most of the issues of post-mortem brain tissue analysis can be addressed by analysis of ante-mortem tissue or CSF samples. The use of ante-mortem tissue for diagnosis of neurological disease is limited to rare neurological specimens. Procter et al. [99] used an assay based on precolumn derivatisation with OPA to study changes in levels of glutamate and aspartate in brain samples from patients with AD. The authors reported that early in AD there was a slight elevation of aspartate in the cerebral cortex and a reduction in the concentration of glutamate of a similar magnitude. They proposed that these changes together with a decrease in glutamine level in CSF and lack of change in the phosphate-activated glutaminase activity of tissue shows an early metabolic abnormality.

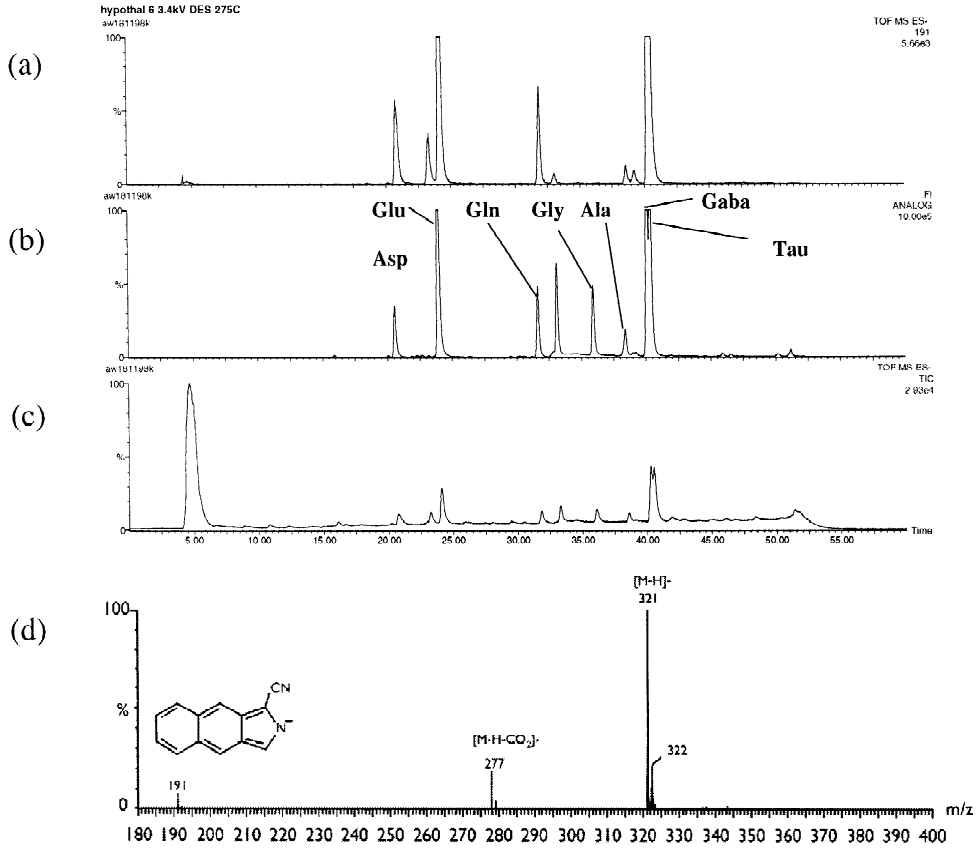

Fig. 3. Chromatograms of a sample of hypothalamus tissue treated with NDA following deproteination and centrifugation: (a) chromatogram extracted from total ion chromatogram of species with fragment ion of molecular mass 191; (b) chromatogram recorded using fluorescence detection in tandem with mass spectrometric detection; (c) total ion chromatogram; (d) mass spectrum of CBI derivative of glutamic acid extracted from total ion chromatogram. All HPLC conditions were identical to those described for Fig. 1.

Analysis of CSF has been used widely to study the changes in neurotransmitter amino acid levels in patients with neurological and psychiatric disorders. Ion-exchange chromatography separation followed by post-column derivatisation with ninhydrin has been used to investigate differences in concentration of neurotransmitter amino acid in CSF and plasma between a control group and patients with AD [100] and those afflicted with PD [101]. The authors reported that in comparison with control group those with AD had higher levels of glutamate and glycine in CSF, higher levels of aspartate and glycine and lower concentration of GABA in the plasma. In the study on PD, the authors found that control group and patients with the disease had similar concentration of glutamate and higher level of glycine in CSF and higher levels of glycine and lower concentration of aspartate in plasma. The precolumn OPA–2-mercaptoethanol assay has also been used to investigate amino acid levels in CSF and plasma from patients with aseptic meningitis [102]. The authors found higher levels of aspartate, glutamate and GABA in CSF of patients in comparison to healthy controls and suggested that this could be used diagnostically in assessing the progression and remission in aseptic meningitis.

Recent studies have focused on the introduction of the in vivo microdialysis technique for the moni-

toring of neurotransmitter amino acids in neurointensive care units with the primary goal of detecting and preventing secondary cerebral lesions [103–105]. Thus, lactic acidosis and release of excitotoxic amino acids are key neurochemical features of secondary brain damage following head injury [106]. Significant elevations of glutamate, aspartate, glycine and the putative glial osmoregulatory amino acid taurine have been reported during jugular venous oxygen desaturation, intractable increased intracranial pressure, and in the case of brain death [107]. Furthermore, a close relationship between cerebral blood flow (CBF) and glutamate release in patients with severe head injury has been demonstrated [108]. Thus, a persistent release of glutamate may be involved in delayed brain swelling (penumbral astrocyte swelling, ionic leakage and calcium entry) and seems to occur simultaneously with CBF levels below the ischaemic threshold of 18 ml/100 g/min. In contrast, in patients without secondary ischaemic complications delayed post-traumatic glutamate release appears to be only transient or does not occur at all. Additional studies have also shown that extracellular levels of glutamate in the subfrontal and medial temporal cortices can reach extremely high levels in patients suffering from aneurysmal subarachnoid haemorrhage [109]. Under baseline conditions, glutamate levels in the human temporal cortex are around 20–24 μM [110]. An increase in glutamate concentrations by about 2–5 μmol/l may result in neuronal damage [111]. Furthermore, glutamate levels in these cortical regions seem to follow the patient's clinical status: the higher the glutamate levels, the higher the neurological and neuropsychological sequelae. Interestingly, hypothermia has been shown to present neuroprotective properties probably by decreasing extracellular glutamate levels (decreased release or increased re-uptake) and reducing the intracellular build-up of calcium [112]. Studies suggest that small decreases in brain temperature may exert neuroprotective effects by a reduction in ischaemia-induced glutamate release [113]. Altogether these studies indicate the value of bedside analysis of amino acid neurotransmitters for the detection of ongoing ischaemic events and the delivery of optimized treatment. Such findings have also obvious implications for drug discovery research.

5. Future developments

The analysis of amino acid neurotransmitters can be carried out using a wide range of separation-based assays. The methods that have been used provide adequate selectivity, sensitivity and precision to allow amino acids to be measured in brain tissue and dialysis samples. However, there is an ever-increasing demand to improve throughput and to obtain unequivocal identification of the analytes in biological matrices. The measurement of amino acids using assays based on mass spectrometric detection provides a means of confirming the identity of analytes. However, there remains a need for the development of rapid LC assays based on mass spectrometric detection. This may be addressed by development of LC assays based on tandem mass spectrometric detection. Although the selectivity obtained using tandem mass spectrometry is very good, analytes have to be resolved from the solvent front to minimise signal suppression. Hence, neurotransmitter amino acids will still need to be derivatised to aid their separation. Also, the mass spectral characteristics of the majority of neurotransmitter amino acids are poor. This can be improved by introduction of a suitable group during derivatisation.

The application of separation-based assays for the diagnosis of neurological and psychiatric disorders is limited. However, changes in levels of amino acid neurotransmitters could serve both as indicators of the disease and its prognosis. Further refinement of recent sampling technologies both at the pre-clinical level, but also in neurointensive medicine will provide enormous potential for revealing the role of amino acid neurotransmitters in normal and pathological processes.

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