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Techniques for neuropeptide determination

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Abstract

Because immunoassay responds to epitopes, and many molecules share the same peptide epitope, it is very difficult to obtain an accurate understanding of peptides, their creation and hydrolysis, in biological systems. Separate-and-detect approaches have merit in that the many active peptides and inactive fragments of a particular system can be separately determined. This review discusses the separation, by chromatography and capillary electrophoresis, and detection, by absorbance, fluorescence, electrochemistry, and immunoassay techniques. When separation pre-concentration is accompanied by laser-induced fluorescence or biuret-based electrochemical detection, nM-pM detection limits are obtained.

Keywords

Chromatography; Electrochemical detection; Electrophoresis; Neuroprotection; Peptides

1. Introduction

The main actors in fast neuronal communication in the brain are amino acids, of which glutamate and GABA (gamma-aminobutyric acid) clearly dominate the scene. Other low-molecular-weight transmitters include acetylcholine and various catecholamines [1].

During the last 30 years, the steadily increasing number of identified neuropeptides that are colocalized with the low-molecular-weight transmitters illustrates that the chemical language of nerve cell communication is far more complicated than originally anticipated. The full physiological function of these neuropeptides is not understood but a general view is that they modulate the metabolism, sensitivity and transcription of their cellular targets *via* G-protein-coupled receptors [2–4].

Many neuropeptides show plastic expression and are upregulated following injury which implies that they may have long-term trophic effects. In a recent review [5] by one of the pioneers in neuropeptide research, Hökfelt suggests: “There is evidence that peptides may exert their main actions when the nervous system is stressed, challenged or afflicted by disease. In fact, this suggests that peptides are important in signaling under these circumstances and that peptidergic communication may be the language of the diseased brain which should make peptidergic mechanisms targets for drug development.” In Alzheimer’s disease, this may be particularly relevant, as neuropeptides, such as somatostatin, neuropeptide Y, corticotropin-releasing factor and substance P, are reduced (whereas others are spared) [6].

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One “hot” area in which neuropeptides may be of high importance is in formation of new neurons in adult life. Interest in such neurogenesis exploded after it was shown that nerve cells are born and survive late in life in human hippocampus [7]. Neurogenesis could allow the brain to respond to challenges from intellectual activity or from injury [8].

The factors, called neurotrophic factors, that regulate the formation of new neurons are far from fully known, but include neuropeptides such as neuropeptide Y. The term neuropeptide implies a neuronal localization and that is incorrect. These peptides have also been localized to various types of glial cells and could thus be released from both neurons and glial cells. Interesting in this respect is that neuropeptides also can modulate glial cell function, for example the expression of proinflammatory chemokines in microglial cells. Such chemokines have been associated with the etiology of neuropathology in conditions such as stroke, Alzheimer’s disease, AIDS, dementia and multiple sclerosis.

It is obvious that the success for peptide-based treatment [9] of neurological diseases must be based on the deep understanding of the neuropeptides that exist in brain and their neuroprotective or neurotoxic effects. It follows that the success of this area in neuroscience relies on correct, sensitive and uncomplicated methodologies for quantitative analysis of neuropeptides.

This short review takes up some of the methodologies in this challenging area of analytical chemistry research. One area that we do not cover and that shows perhaps the greatest development in recent years is detection by various mass spectroscopic (MS) techniques. For MS techniques, the reader is referred to Hummon et al. in this issue [55] and recent reviews [10–13].

2. Separation of neuropeptides

Peptides can be separated with reversed phase liquid chromatography (RP-LC), which is based on the hydrophobicity of peptides. Early work [14–17] showed that the retention of small peptides of up to 20 amino acids can be predicted to a degree based on the simple sum of the hydrophobicities of the individual amino-acid side chain. The peptide backbone itself contributes little to the retention. Larger peptides, such as insulin, do not show such predictability, probably because the individual amino acids cannot simultaneously interact with the stationary phase.

As a practical matter, for C-18 stationary phases, the preferred eluents are acidic (trifluoroacetic acid and formic acid) and use acetonitrile as the organic cosolvent. Because of the very large increment in hydrophobicity caused by the addition of, for example, a benzyl group in a phenylalanine, it is difficult in general to take advantage of isocratic separations. Most often, gradient elution is required. Gradient elution has the very useful advantage of allowing preconcentration. As many samples in neurochemical investigations are in the μL range or lower, preconcentration requires the use of microcolumns [18].

Both large and small peptides can be separated by capillary electrophoresis (CE) [19]. In fact, because the basis of the separation is not at all related to hydrophobicity, CE is, in many ways, ideal for separating peptide mixtures that may consist of at least some large (> 20 aa) peptides [20]. Acidic conditions are best for peptides. Wall effects are common, and the highly acidic buffers used minimize this practical problem.

Capillary electrochromatography (CEC) can also be effective for peptides [21]. Recent work on monolithic systems [22] demonstrates that the somewhat hydrophobic acrylates that are quaternized work well for peptides. Including a cellulose acrylate ester in the *in-situ* polymerization to create the monolith leads to even more separating power. Others have

similarly found that a not-too non-polar, cationic stationary phase works well for peptide separations. Comparison of CEC with CE under similar conditions has shown that the chromatographic process is important to the separation.

3. Detection of neuropeptides

3.1. UV absorbance

In general, detection of neuropeptides in brain samples by UV (ultraviolet) absorbance after HPLC (high-performance LC) or CE separation is hampered by levels of neuropeptides below the detection limit, interactions from species in the matrix and/or difficulties in finding separation strategies that are compatible with UV absorption. Nevertheless, for preparative work and for studies on degradation of neuropeptides *in vitro*, detection at 210 nm (peptide bond) or 280 nm (if an aromatic amino acid is present) may be sufficient [23].

3.2. Fluorescence

For fluorescence detection, there are in theory and practice several different principles [24], namely pre-column and post-column derivatization, and using native fluorescence. Pre-column derivatization resulting in detection limits in the lower pmol range has been used for lysine-containing neuropeptides employing reagents, such as fluorescamine [25], naphthalene-2,3-dicarboxylaldehyde/cyanide [26] and 9-fluorenylmethyl chloroformate [27]. The major drawback with pre-column derivatization is that several reaction sites may be present and that can result in multiple peaks for one neuropeptide and possible quenching of the fluorescence signal. Post-column derivatization will at least overcome the problem with multiple peaks. Post-column reactions inevitably add to band spreading, so it is remarkable that post-column fluorescent derivatization has been carried out, even in CE [28].

In view of the low concentration of neuropeptides in all kinds of brain samples, it is obvious that these kinds of strategies put a high demand on the (pre)separation of the neuropeptides from interfering species.

Finally, more attention is being paid to using native fluorescence detection [29,30]. While instrumentally more complex than procedures that use derivatization, problems of multiple derivatives (pre-column) and band spreading (post-column) do not occur with this approach.

3.3. Electrochemical detection

Determination of neuropeptides by employing electrochemical (EC)-detection can be based on either inherent EC active groups within the neuropeptide or it can be achieved by various derivatizations. Inate redox-active functionalities include tyrosine, tryptophan, methionine and cysteine residues [31,32].

One problem with cysteine residues in neuropeptides is that samples will contain a mixture of reduced, oxidized and mixed disulfides of the neuropeptide. To avoid multiple peaks some kind of pre-analysis treatment is thus necessary.

In order to reduce the EC interferences from oxidation of matrix solutes at the working electrode, a “cleaning” potential on an upstream electrode can dramatically increase the performance of EC detection.

One way to increase the selectivity and sensitivity of neuropeptide determination by EC is, in analogy with fluorescence, to derivatize the neuropeptides with an active redox-group. One such agent is copper which binds to the peptide bond [33]. This technique can be used both pre-column [34,35] and post-column [36–38]. Fig. 1 demonstrates the concept. Peptides are separated by gradient elution RP-LC. A post-column addition of Cu(II) in basic tartrate solution

causes the rapid formation of electroactive Cu–peptide complexes that are oxidized at an anode. The Cu(III) reaction products may be detected at following cathode. The ratio of current at the downstream cathode to the current at the upstream anode is called the collection efficiency. It is typically in the range 0.25–0.3; however for some peptides that are not stable in the Cu(III) form, it can be lower.

Fig. 2 shows results from the pre-column derivatization of peptides and separation with preconcentration by microcolumn chromatography. The detection limits in these experiments are low enough to quantitate resting concentrations, and to discern changes in peptide concentrations over time. The advantage of the technique is that it leads to EC active complexes from all peptides investigated so far. This includes many bioactive, large peptides (such as insulin), short dipeptides, disulfide-bridged peptides (such as oxytocin), and peptides with no primary amine with detection limits in the low nM range [39,40]. The rate of reaction of N-formyl-peptides (with the primary amine formylated) with copper reagent is slower than when the primary amine terminus is free [41]. However, this does not prevent their determination. For example, thyrotropin-releasing hormone (TRH, pEHP) has no primary amine, yet it can be determined in dialysate and brain homogenate [42].

3.4. Coupling EC and fluorescence detection

In a novel method, the selectivity of electrochemistry is coupled with the advantages of fluorescence by a post-column reaction with fluorogenic reagents [43,44]. Ru(II) and Os(II) bipyridine complexes are fluorescent, but their (III) oxidation state analogs are not. At the same time, the M(III) compounds are good oxidants. Thus, combining a chromatographic effluent with an M(III) reagent leads to the production of the fluorescent M(II) complex as a direct result of the presence of an oxidizable species.

Fig. 3 shows the general scheme of this approach. The electroactive fragments of dynorphin A are separated and quantitated. It is interesting that the sensitivity of the method is directly related to the number and type of oxidizable functional group in the peptide.

3.5. Post-column reactions

It is necessary to carry out post-column reactions in many of the cited works. Microcolumn separations place severe demands on the volume and the characteristics of post-column reactors. A novel approach, based on heat-shrink fluorinated ethylenepropylene/polytetrafluoroethylene tubing, shows promise for post-column mixing and detection [45–47]. Channels as small as 13 μm in diameter can be created in this inert material. Devices such as “Y” and “T” mixers and EC cells can be created.

The Cu-based approach works best for tripeptides and longer peptides [48]. Although detection limits are a little poorer, dipeptides can be determined either with post-column reaction [49–51] or through the use of a modified electrode.

3.6. Radioimmunoassay

For detection of neuropeptides in all types of brain samples, the technique most used by far is radioimmunoassay (RIA). The technique, which normally is based on competition of native and radioactive neuropeptides to antibody sites, is very sensitive, down to low fmol levels, and relatively simple at comparatively low cost.

The specificity is based on the antibody and therefore cross-reactivity with various neuropeptide fragments and pre-neuropeptide forms may give incorrect data from a quantitative point of view.

To avoid major interferences and reduce the level of cross-reactivity, RIA is often employed after prior separation by HPLC, which makes it a rather time-consuming but unambiguous technique [18,52,53].

3.7. Immunoaffinity separations

Antibodies to neuropeptides can be attached to column packing material or fused silica walls [54] and used for separation and measurements of several neuropeptides in the presence of interfering substances in a single sample. Coupling 30 such capillary immunoaffinity columns in series, it was possible to analyze simultaneously several pre-column derivatized cytokines and four neuropeptides at pM concentrations using laser induced fluorescence.

4. Determination of neuropeptides in real samples

Whatever technique to be used, neuropeptide analysis is rather challenging from a sample-preparation point of view. The neuropeptides can adsorb to plastic and glass surfaces and to sites in the column. Chemical decomposition can occur in certain buffers and not in others, and the sample may contain known and unknown peptidases that may be difficult to control for completely. All these aspects need thorough consideration before real samples can be analyzed successfully.

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Dual electrochemical detection of 10 non-electroactive peptides as copper(II) complexes

Figure 1.

Reversed phase gradient elution separation and detection of 10 non-electroactive peptides (name or primary sequence): 1) VGVAPG; 2) GGFL; 3) speract; 4) fibrinopeptide A; 5) octadecaneuropeptide; 6) buccalin; 7) N-f-MLF; 8) ALILTLVS; 10) secretin. The scales of the two chromatograms are not the same; the cathode scale is 3.3 times more sensitive than the anode scale, so that, for normal collection efficiency of about 0.3, the peaks will appear the same size.

Figure 2.

In-vivo monitoring of vasopressin (A) and bradykinin (B) in the rat SON with 5-min sampling frequency. Bar indicates stimulation by application of high- K^+ buffer (corrected for dead time of system). Each point is the average from four animals with ± 1 standard deviation given by the error bar. [Reprinted with permission from [34]. Copyright (1999) American Chemical Society].

Gradient Elution of Dynorphin A and Fragments

Figure 3.

Separation and detection of dynorphin A fragments. Reagent: 10 μM Ru(bpy)_3^{2+} /0.1% trifluoroacetic acid/0.1 M NaClO_4 /acetonitrile oxidized to Ru(III) at 1.6 V versus Ag/AgCl . [Reprinted with permission from [43]. Copyright (1999) American Chemical Society].