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Separation and detection techniques for peptides and proteins in stability research and bioanalysis☆

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Abstract

In this paper, a brief overview of the most commonly used methods for the separation and analysis of peptides and proteins in stability and bioanalysis studies is presented. To investigate the physical stability of peptides and proteins, size-exclusion chromatography and electrophoretic separation techniques are being used, apart from several other methods. To determine the chemical stability of these compounds, separation systems are also important, with informative detection modes, such as various spectroscopic detections, electrochemical detection and mass spectrometric detection. For the bioanalysis of peptides, separation is the most important factor, while the detection must be done at the highest possible level of sensitivity.

Keywords: Peptides; Proteins

1. Introduction

The analysis of proteins and peptides has been a challenge to researchers for many years. Initially, attention was focused on the isolation, characterisation and properties of endogenous proteins and peptides. Analysis on various scales was required, from procedures suitable for complex biological matrices, such as plasma and tissues, to the level of single-cell analysis. Each of these approaches requires analytical methods that are suitable to its specific problems, such as high specificity, high resolution separation or high sensitivity. A modern development in this respect is the peptide profiling of biological systems in order to establish the ''normal'' profile of a healthy system and to study alterations in these profiles in the case of disease. For this approach, coupled techniques must be applied [1–4].

With the development of new peptide and protein drugs originating from recombinant DNA technology, the need emerged to characterise these drugs, to establish their stability for proper formulation and to develop methods for their bioanalysis. Our research group focuses on the development of appropriate analytical methods for these compounds and, in this paper, some examples of methods for stability tests as well as for the bioanalysis of peptides and proteins will be given. The analytical methodology generally

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involves the development of appropriate separation systems and detection techniques.

2. Stability of peptides and proteins

The instability of peptides and proteins involves both physical and chemical degradation. For both types of processes, one has to apply specific analytical methods.

The physical instability of proteins and peptides involves changes in the higher order structures of these compounds, provided that they possess these structures, e.g., proteins and polypeptides with higher molecular structures. The chemical composition is not altered. The changes involve unfolding of the protein and aggregation of the compound. They can be caused by various factors, such as temperature, ionic strength, pH, etc. In addition, specific interactions with so-called denaturing factors might be the reason. Both unfolding and aggregation can be monitored by various analytical methods.

Unfolding of proteins results in the total disruption of the tertiary and often the secondary structure of the compound [5], resulting in exposure of the lipophilic parts to a more hydrophilic environment. The unfolding can be either partial or complete, depending on the conditions [5,6]:

—partial: high temperature, high ionic strength, extreme pH;

—complete: denaturing factors: urea, guanidine HCl. The unfolding can follow two pathways:

—a co-operative two-state transition [Eq. 1]:

$$F \leftrightarrow U \quad (1)$$

—a transition from the folded to the unfolded state through an intermediate state [Eq. 2]:

$$F \leftrightarrow I \leftrightarrow U \quad (2)$$

Often, the properties of these states exhibit a high content of secondary and tertiary structures. They are called molten globule states and show different properties both from the folded and unfolded states [6].

Various analytical techniques can be used to study the protein's unfolding process. Since the process is endothermic, thermoanalytical methods like differential scanning calorimetry (DSC) can be used. The energy uptake is maximal at the transition [5,7–14].

The loss of higher structures changes the spectroscopic properties of proteins. Fluorescence and CD spectroscopy can be used to monitor the unfolding process. For instance, the increased exposure of Tyr and Trp to a hydrophilic environment results in a redshift in the fluorescence maximum, while the intensity decreases [10,15,16]. In addition, indirect methods, such as the increasing fluorescence intensity of 1-aminonaphthalenesulfonate (ANS) when in contact with the more lipophilic parts of the molecule [17], are suitable.

Due to the loss of specific orientation of the aromatic groups on unfolding, the ellipticity changes, which makes the use of circular dichroism (CD) possible [10,15,16,18–25].

Fourier transform infrared (FT-IR) and ^{1}H-NMR can be used to study the changes that occur during unfolding. In particular, 2D- and ^{1}H-NMR are very useful in monitoring the process. The unique magnetic environment in the closely packed folded structure is the cause for distinct chemical shifts, which change on unfolding [26–29].

Separation techniques are also suitable. Since proteins, on unfolding, change in size and shape, chromatographic and electrophoretic techniques can be used. Size-exclusion chromatography (SEC) [13,24,30] and electrophoresis are suitable techniques. In SEC, for instance, the folded protein exhibits a larger elution volume, while in electrophoresis, the unfolded protein has a longer migration time.

Another possibility is the use of urea-gradient gel electrophoresis. At low urea concentrations, the protein migrates with its native mobility, whereas at high concentration, the migration velocity is that of the unfolded form. The urea gradient is established in a polyacrylamide gel. The transition of the protein can be followed in the intermediate areas [31].

Aggregation of proteins results from hydrophobic interaction between partly unfolded proteins during the formation of large soluble or insoluble structures [32,33]. The most important change in properties are alterations in size and the formation of precipitates. Hence, techniques such as SEC and sodium-dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) can be used to monitor the process. Other

useful techniques are measuring turbidity, equilibrium dialysis and surface plasmon resonance (SPR).

The chemical instability of peptides and proteins involves changes in the primary structure of the compound, due to chemical degradation of this structure. A number of degradation reactions can be distinguished, according to a review by Manning et al. [5], such as oxidation/reduction, deamidation, hydrolysis, racemisation, arginine conversion and β-elimination.

Oxidation is one of the major degradation pathways for proteins and peptides. Sidechains of various aromatic amino acids, such as His, Tyr, Trp and also Met and Cys can be oxidised. Usually, more hydrophilic products are formed, with the exception of the dimerisation of Cys- and Tyr residues leading to more hydrophobic products [5]. In addition, changes in spectroscopic properties can be observed, such as fluorescence changes on oxidation of Trp, Tyr and Phe [34–36]. The same goes for changes in electrochemical properties on oxidation of His and Phe residues. Of course, mass changes can be observed, due to the introduction of oxygen into the molecule during most oxidation reactions.

As a result of these changes, most oxidation reactions can be monitored with RP-HPLC, wherein most oxidation products elute prior to the native proteins, with the exception of the dimers. The detection mode can vary according to the nature of the reaction. UV is the most widely applicable mode, however, it is also the least specific. Fluorescence [35,37,38] and electrochemical detection (ECD) [39] are more informative for Trp or His. Mass spectrometry (MS) or tandem MS, either off-line or on-line with the separation system, gives information on the molecular mass and fragmentation pattern of the products. In some cases, direct monitoring of the process is also possible with these techniques.

Sometimes, the nature of oxidation products can be determined after specific cleavage of certain amide bonds in the protein. In this example, the protein recombinant methionyl human granulocyte colony stimulating factor (G-CSF), prior to and after oxidation with hydrogen peroxide, is cleaved with cyanogen bromide. The methionine residues, of which G-CSF has four, are specifically cleaved by this compound. Fig. 1 shows four degradation products of G-CSF, while in Fig. 2, the cleavage pattern of the parent compound is depicted. A number of fragments can be seen. After oxidation of G-CSF, following isolation of the oxidation products and reaction with cyanogen bromide of every individual product, some of the fragments can no longer be observed, meaning that the methionine residue involved has been oxidised and cannot be cleaved anymore. Using this approach, the structures of all four oxidation products could be established [40].

Sometimes, aggregate formation occurs, due to Cys or Tyr oxidation. The process can be monitored with SEC and electrophoresis.

Reduction of proteins and peptides results in disruption of disulfide bonds and, consequently, changes in the three-dimensional structure of the compounds [41–43]. In smaller peptides, this reduc-

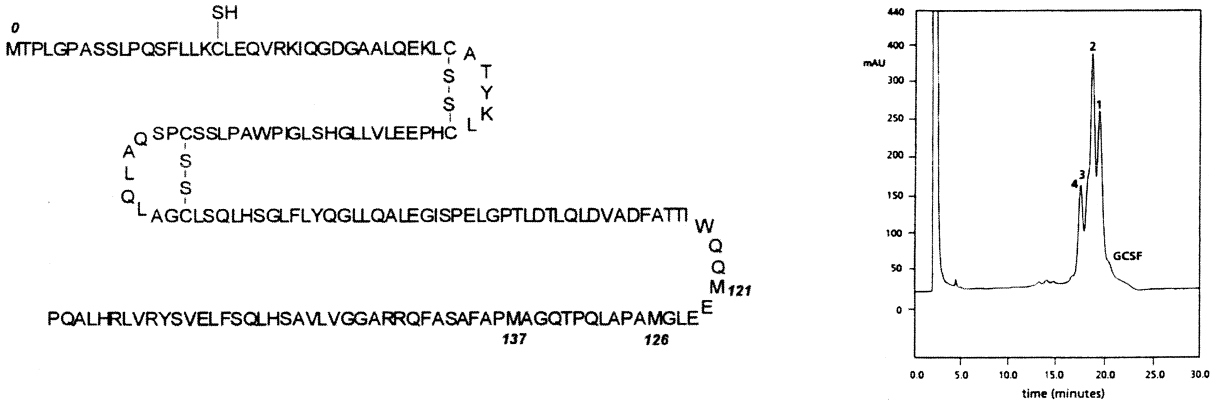

Fig. 1. RP-HPLC chromatogram of oxidised *r*-metHuG-CSF (GCSF). Peaks 1–4 are oxidation products.

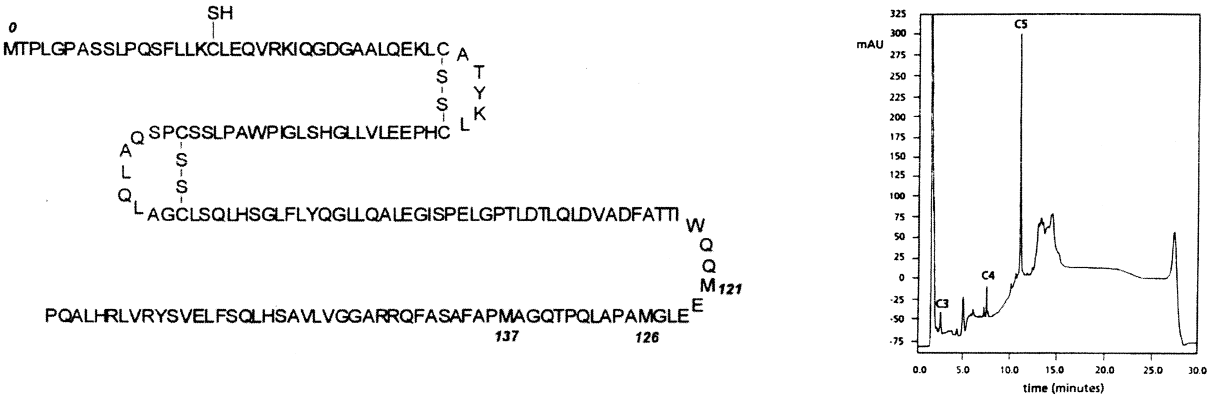

Fig. 2. RP-HPLC chromatogram of cyanogen bromide cleavage of *r*-metHuG-CSF [41]. C3, C4 and C5 are fragments after cleavage.

tion leads to more hydrophilic products. Both RP-HPLC and electrophoresis are suitable techniques to monitor the process.

An example of the separation of reduction products from a native protein is shown in Fig. 3. Reduction of two disulfide bonds in G-CSF leads to a number of products that can be separated by RP-HPLC in a gradient system [44].

Deamidation of C-terminal amide functions in peptides results in the introduction of a free carboxylic acid group. This process is accompanied by changes in polarity and hydrophobicity, mass and charge. During the process ammonia is also formed. All of these changes can be used to monitor the deamidation process. Changes in polarity and hydrophobicity can be followed using RP-HPLC, either isocratic or in a gradient system. Electrophoretic separation can also be performed, since, on deamidation, mass–charge ratios change due to the introduction of a charged carboxylic group. An example of both separations is given in Fig. 4 [45], where the deamidation in acidic media of a substance P antagonist is depicted. The RP-HPLC chromatogram shows a more polar product after deamidation, while the electropherogram shows that the deamidation product has a lower migration velocity, due to a lower mass–charge ratio.

Hydrolysis of proteins and peptides occurs at the amide bonds in the peptide backbone and results in a number of lower-molecular-mass compounds in which free carboxylic acid groups and amino functions have been introduced. In particular, Asp-derived amide bonds are sensitive to hydrolysis [5,46]. Hydrolysis results in changes in mass, size and

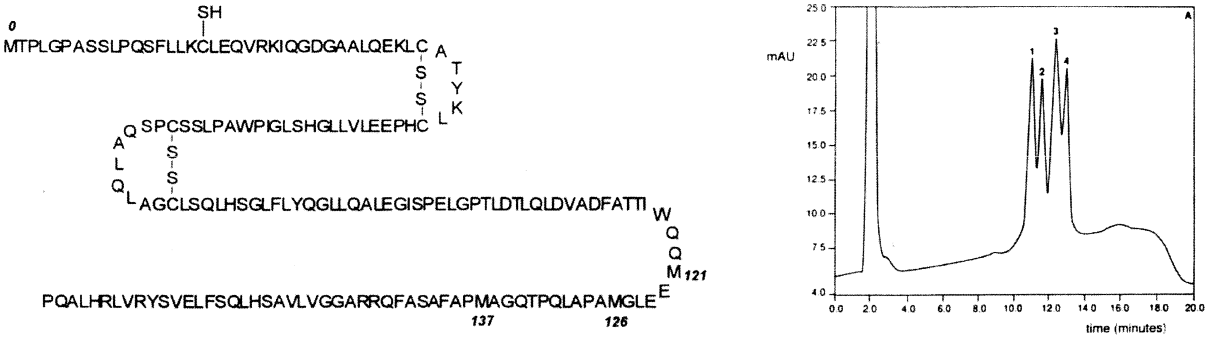

Fig. 3. RP-HPLC chromatogram of reduction products of *r*-metHuG-CSF [45]. Peak 1 is the parent compound, peaks 2 and 3 are products with only one disulfide cleaved, and peak 4 is the fully reduced product.

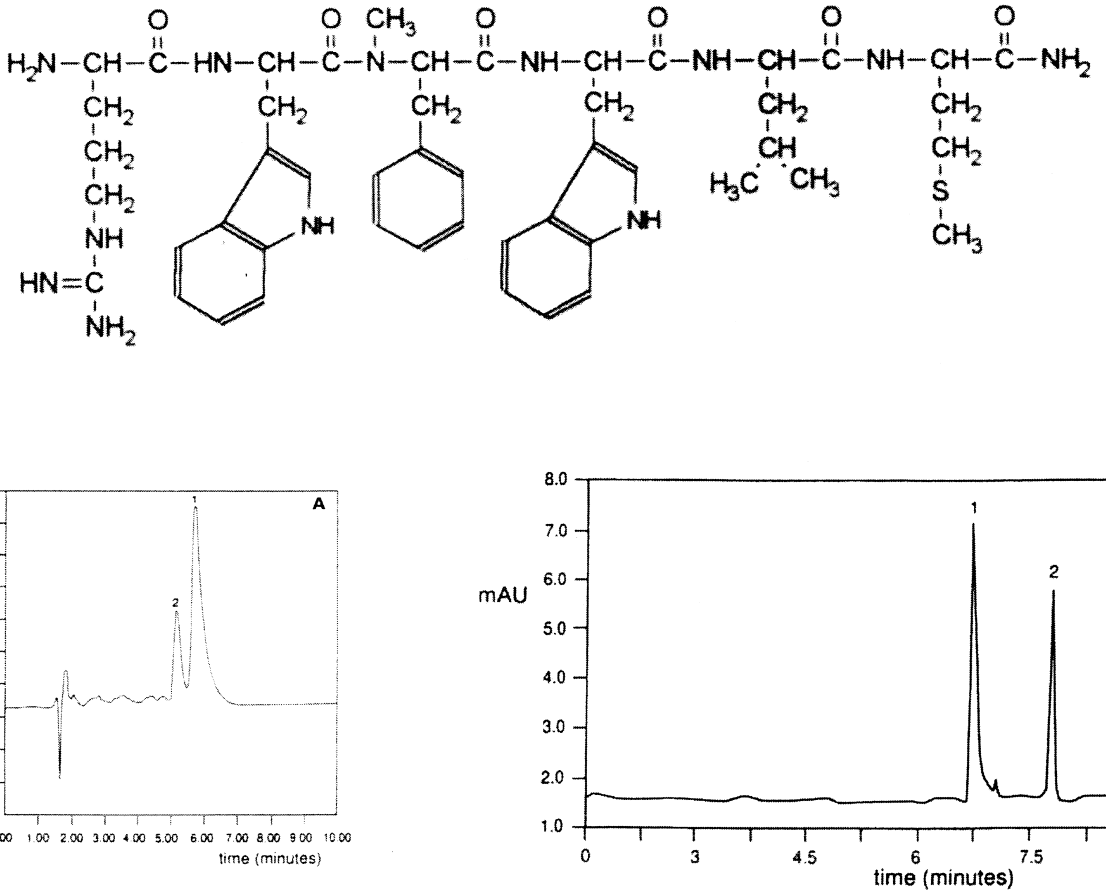

Fig. 4. RP-HPLC chromatogram (A) and electropherogram (B) of the substance P antagonist G (1) and its deamidation product (2) [46].

shape, charge, hydrophobicity and polarity. Sometimes UV absorption and fluorescence properties also change. Obviously, RP-HPLC and electrophoresis are the appropriate separation techniques, with a number of detection possibilities.

Racemisation of peptides is another important mechanism for degradation. The removal of the α-methine hydrogen by an hydroxide ion is the initial step. Stabilisation of the resulting carbanion by electron-withdrawing substituents, such as the side chains of Tyr and Phe, influences the rate of the reaction. The formation of cyclic intermediates, formed by Asp and Glu, also increases this reaction rate. On racemisation of peptides and proteins, diastereomers result that have different polarities and hydrophobicities. The reaction, being carried out in water, does not result in mass and charges changes, unlike the situation in deuterated media where a mass increase of one occurs and the magnetic properties change.

Changes in optical-rotation only indicate whether racemisation occurs or not. Peptide and protein racemisation, resulting in the formation of diastereomers, can be monitored using achiral RP-HPLC. The location of the racemisation site can sometimes be established after complete hydrolysis of the compound and analysis of the resulting amino acid mixture for D-amino acids.

An example is given in Fig. 5, where the racemisation of a substance P antagonist is monitored using chiral GC [45]. The chromatograms, after degradation of the compound and complete hydrolysis of

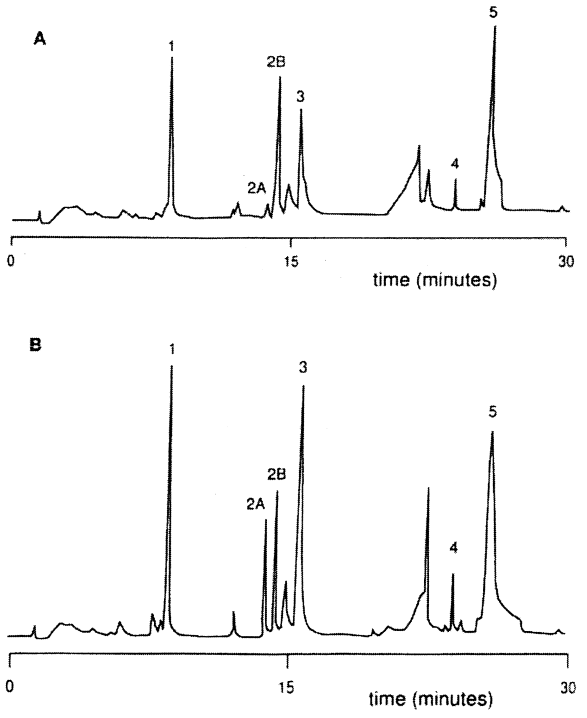

Fig. 5. Chiral GC chromatograms of hydrolysed substance P antagonist G samples after degradation in acidic media (A) and degradation in alkaline media (B). Peak 1, L-Leu; peak 2A, D-Met; peak 2B, L-Met; peak 3, L-MePhe; peak 4, L-Arg and peak 5, D-Trp [46].

the degradation product, clearly show that racemisation only occurs at the methionine residues. Of course, information on the racemisation, obtained with this technique, is only qualitative.

Arginine conversion into ornithine and citrulline and β-elimination have hardly been studied and will not be discussed here.

3. Bioanalysis of peptides

Since the development and therapeutic application of peptides and proteins resulting from recombinant DNA technological processes, the bioanalysis of these compounds has become increasingly important. Generally, this class of drugs is active in rather low concentrations, which makes analysis in biological matrices tedious. Moreover, structural similarities between the drug and endogenous compounds complicates the distinction, and separation, between the two. However, the bioanalysis of these drugs is important for pharmacokinetic studies and therapeutic drug monitoring. Usually, immunoassays are employed that lack sufficient selectivity. More selective approaches involve the separation of proteins from interfering endogenous material and metabolites. Problems in the bioanalysis of peptides and proteins are encountered in the concentration stages and the separation systems.

RP-HPLC has been used in a vast number of studies, being a robust and easily applicable method. Depending on the biological matrix, prechromatographical solid-phase extraction (SPE) has to be performed to remove interfering endogenous substances.

SPE has distinct advantages over precipitation procedures for removing interfering serum proteins, since concentration steps can easily be included. For cationic peptides, SPE is best performed using weak cation-exchange cartridges. After evaporation of the eluent, the residue can be subjected to RP-HPLC. A separation technique that seems to be very promising is CZE, with its high resolution power. CZE is very suitable for peptides, since these compounds have a number of acidic and basic groups and are charged at various pH values. A disadvantage of CZE, however, is its poor concentration sensitivity, due to a low sample loadability and short optical pathway. Several strategies can be explored to overcome these problems. The use of Z-shaped or bubble detection cells may improve the detector's sensitivity [47,48], or pre-, on- or postcapillary derivatisation can be performed [49]. These approaches often lead to laborious procedures. Concentration procedures also have been tried, using field amplified sample injection (FASI) [50,51], isotachophoresis–CZE (ITP–CZE) [52–54] or chromatographic preconcentration steps [55]. These procedures usually lead to non-robust assays.

In our group, we investigated the possibility of using transient ITP–CZE (tITP–CZE), a procedure where, in a standard fused-silica capillary, ITP is performed using the total volume of the capillary. The concentration step was done at high voltage (+30 kV) with backpressure (−100 hPa). In this way, the terminating electrolyte was prevented from

entering the capillary, and the sample zone, during concentration, was brought back to the inlet. After concentration, the inlet vial was replaced by the leading electrolyte buffer, which also served as the background electrolyte during the subsequent CZE analysis. A combination of off-line SPE, followed by either RP-HPLC or tITP–CZE yielded a sensitivity in the nanogram region, as is shown in Fig. 6 for gonadorelin and angiotensin II [56]. For anionic peptides, dynamically coated capillaries with a quaternary ammonium compound were used. The electroosmotic flow (EOF) in these cases was reversed and, thus, directed to the outlet vial with reversed polarity, preventing the anionic species from entering the inlet vial again.

At present, we are investigating the possibility of off-line SPE analysis of plasma samples, followed by a derivatisation reaction and either RP-HPLC or tITP–CZE with fluorescence detection to further improve the sensitivity of the assay. We are also attempting to connect the MS detection system to the separating systems, not only because of improved sensitivity but also for use in those cases where separation in the previous system was not complete.

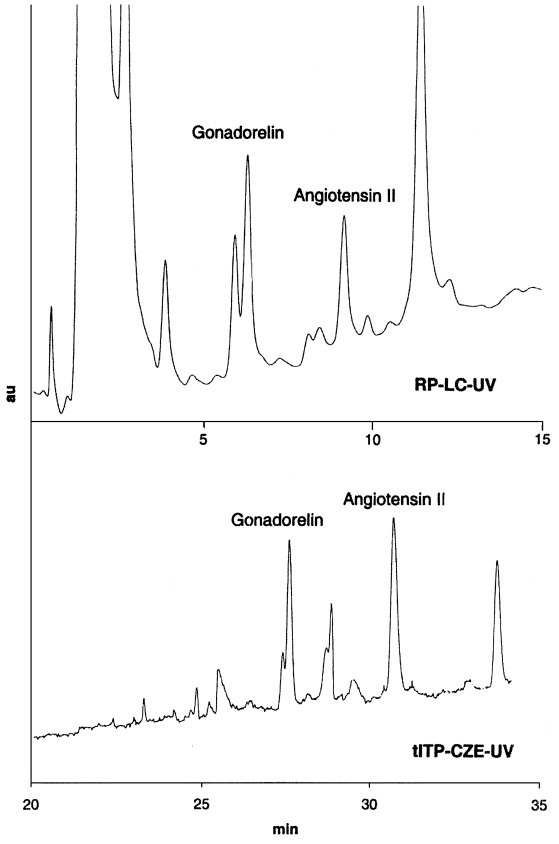

Fig. 6. tITP–CZE–UV and RP-HPLC-UV analysis of an extracted gonadorelin–angiotensin II plasma sample (60 ng/ml) [58].

4. Detection techniques in protein and peptide research

The analytical methodology for monitoring protein and peptide stability and bioanalysis involves a number of physicochemical methods in physical stability research, whereas in chemical stability studies and bioanalysis, a combination of a separation technique and a suitable detection technique is usually employed.

For physical stability testing, a number of techniques have been described, such as fluorimetry and CD spectroscopy to monitor the unfolding process [10,15,16]. The energy uptake when proteins unfold can be studied using thermoanalytical methods like DSC [5,7–14]. Other techniques to monitor these processes include FT-IR and ^{1}H-NMR [26–29], while aggregation and precipitation can be monitored using SEC and SDS–PAGE. Recently, SPR was introduced as a suitable detection technique [57].

To test chemical stability and in the bioanalytical research of proteins and peptides, the main separation techniques used are RP-HPLC and CZE. The most frequently employed detection technique is UV absorption at 214 nm, which is the absorbance maximum of the amido function of the backbone. Fluorimetry can be employed either when native fluorescing functions, like Trp and Tyr, are present [34–38] or after derivatisation with a suitable fluorophore. In these cases, ECD also offers possibilities [39]. MS and tandem MS can provide information on the molecular mass and the fragmentation patterns of peptides and proteins, either off-line or on-line with the separation system.

Reviews on analytical approaches used in research into the physical and chemical stability of proteins and peptides have been published recently [58,59].

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