On-line multidimensional separation systems for peptide analysis

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On-line multidimensionale scheidingssystemen voor de analyse van peptiden

(met een samenvatting in het Nederlands)

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Aim and Scope

Today, there is an increasing interest in selective and sensitive analysis of proteins and peptides with a relatively high speed. The study of protein and peptide profiles of biosystems and the modifications of these profiles in case of certain diseases is important. For the early diagnosis of these diseases the fast, selective and sensitive detection of specific peptides or proteins in plasma or other biological samples is essential. Another example is the increasing application of new potent peptide and protein drugs and, hence, the bioanalysis of these compounds.

Low concentrations of peptides and proteins can be determined with a wide variety of methods. However, as structural differences between endogenous and exogenous peptides and proteins, used as drugs, sometimes are generally small, the conventional one-dimensional bioanalytical methods often do not have sufficient selectivity. On-line coupled multidimensional analysis has the advantage of enhanced selectivity with a high speed. In the first chapter of this thesis several strategies for the on-line multidimensional analysis of peptides and proteins in biological samples have been described. An overview of the existing approaches to couple on-line different separation techniques for the determination of proteins and peptides includes the more conventional liquid chromatography (LC)-LC coupling, as well as the on-line LC-capillary electrophoresis (CE) coupled systems and the strategies for on-line CE-CE coupling. Special attention is paid to the interface between the dimensions as well as the design of the coupled systems.

CE is very suitable for the separation of peptides and proteins. The main advantage of CE over reversed-phase liquid chromatography (RPLC) is the much higher separation efficiency. However, especially when trace-level concentrations have to be determined, the analyte detectability in units of concentration is relatively low. Consequently, using either one-dimensional or on-line coupled CE, analyte enrichment prior to CE separation is often necessary. Therefore, an overview of the possibilities of on- or in-line preconcentration procedures in combination with a CE separation, focused on the determination of peptides and proteins, is also included in the thesis. The methods are categorized in electrophoresis-based and chromatography-based preconcentration and chromatography-based preconcentration is further subdivided in procedures with low specificity and high specificity.

In an aim to improve the already available multidimensional methods for peptide analysis, two new two-dimensional on-line systems have been developed and optimized: a size

exclusion chromatography (SEC)-RPLC system and a SEC-CE system, both with UV detection. In both systems the heart-cut principle is used to isolate a fraction, containing six enkephalin peptides as target compounds, from proteins and other interfering substances by SEC. Subsequently, this fraction is transferred to the second (RPLC or CE) dimension and its components are separated. In the SEC-RPLC system a loop is used for transfer of the enkephalin-containing fraction, while in the SEC-CE system the enkephalins are trapped on a RPLC micro column and analysed by CE after elution. Both systems allow the injection of cerebrospinal fluid (CSF) samples without pretreatment and are suitable for the determination of exogenous enkephalins in CSF. In the general conclusions of this thesis preliminary experiments with more sensitive detection methods, such as fluorescence and MS detection are described in order to illustrate the possibilities and difficulties of the coupling with these detection modes. Some recommendations are given for the achievement of a higher sensitivity.

Chapter1

On-line multidimensional liquid chromatography and capillary electrophoresis systems for peptides and proteins

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Abstract

Peptides and proteins are gaining increasing attention in biosciences and, consequently, in analysis. This overview highlights the different approaches to couple on-line various separation techniques for the determination of proteins and peptides. The first section discusses the liquid chromatography (LC)-LC coupling, the second one reviews the on-line LC-capillary electrophoresis (CE) coupled systems and the third section summarizes the strategies for on-line CE-CE. The advantages, disadvantages, most relevant difficulties and particular systems for on-line coupling are discussed. Special attention is paid to the interface between the two dimensions. Applications are summarized in tables and a few typical examples are discussed.

Many multidimensional separation methods are available, and it is demonstrated that peptide and protein mapping, or quantitation of proteins or peptides in various samples (aqueous solutions, cells, plasma) require different coupled systems. For mapping a semi-quantitative detection is often sufficient, while comprehensiveness is very important. For quantitation of a certain peptide or protein at a low concentration level a validated method should be used, while a heart-cut transport of the first dimension to the second one can offer sufficient selectivity. The combination with mass spectrometry as part of the total system is stressed and illustrated.

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1. Introduction

For the bioanalysis of peptides and proteins in biological samples, one-dimensional separation methods often do not have sufficient selectivity. In these cases, multidimensional methods may offer higher selectivity [1-7]. An increase in selectivity of a multidimensional system can only be obtained when the dimensions are based on different separation mechanisms. The second dimension of a multidimensional system should not reverse the resolution achieved by the previous one [4]. Adequate interfacing is needed to transport the various fractions from the first to the second dimension.

The interfacing for multidimensional column separation systems can be performed in various ways. The effluent of the first separation system can be transported to the next system manually (off-line) or automatically e.g. with the aid of a robot (at-line), or via connecting tubing and/or a valve that directly transports a stream of liquid to the next system (on-line). In general, the on-line combination of separation systems enable a significantly faster analysis of a complex matrix in comparison with an off-line or at-line combination.

As an on-line interface in a coupled system generally a loop or a column is used to trap a fraction of interest, where a switching valve is employed to inject this fraction into the next separation step [8]. If the separation of the complete sample in all dimensions is achieved, the system is considered to be completely multidimensional or comprehensive [9]. In this case the time required for performing a separation in the second dimension must be the same as for filling e.g. a loop with effluent from the first dimension. Such a comprehensive on-line system can only be achieved if adjustments in the analytical dimensions (flow rates, column dimensions) of the system components can be made, because the second dimension has to perform separations much faster. An important reason for the development of comprehensive multidimensional separation methods is the challenge to establish protein profiles of cells and other biological samples. These profiles are very important for research in the field of proteomics. It should be noted that in proteomic research multidimensional column systems are mainly used for the separation of peptide mixtures after digestion of proteins. Recently a review has been published by Wang and Hanash [10] about multidimensional LC-based separations in proteomics, including off-line techniques as well.

If a coupled system separates only a fraction, retrieved from the first dimension, in the second dimension it is called a heart-cut system [8]. These systems are not comprehensive, however these separations are important for the determination of structurally closely related peptides and proteins in biological samples such as plasma and tissues, which require analytical methods with high

selectivity and sensitivity. Multidimensional heart-cut systems often can offer sufficient selectivity and sensitivity for the assay of these compounds in a relatively short time of analysis [11-17].

Two-dimensional gel electrophoresis is widely used for protein profiling [18-21] and usually uses isoelectric focusing (IEF) followed by sodium dodecyl sulfate polyacrylamide gel sieving electrophoresis (2D IEF-SDSPAGE). These two are orthogonal separation modes. For this reason it provides high resolving power for the separation of complex samples containing proteins [3, 4]. However, 2D gel electrophoresis has some limitations: extensive sample handling, time consuming, difficult to automate, decreased resolving power for proteins with a molecular mass of < 15 kDa as a result of their high mobility in the gel and the gel can not be coupled on-line to a mass spectrometer (MS). Moreover, the quantitation in 2D gel electrophoresis is also a problem. After staining the spots can be detected, but a direct (semi)quantitative detection method is not applicable [18-21]. Using a off-line multidimensional column system direct quantitative detection with UV absorbance, Laser Induced Fluorescence (LIF) or MS detection is possible. Therefore, off-line multidimensional LC systems have often been applied for profiling in proteomics research [22-25]. If the multidimensional column system is on-line coupled there are additional advantages: limited loss of analyte due to adsorption to the walls of vials or pipettes, which often occurs in analysis of peptides or proteins in the pmol range [26]. Moreover, increased reproducibility of the method can be achieved due to the lack of manual sample treatments necessary for the transfer of the analytes from the first to the second dimension [8]. On-line coupling is also easy to automate [8] and operator involvement is reduced [26]. Disadvantages of on-line coupling are the increased complexity of the system which may result in a higher chance of peak broadening due to dead volumes [8], and the necessity for relatively long retention or migration times in the first dimension to enable the (comprehensive) coupling [4].

The present review focuses on the development of various on-line multidimensional separation systems for the determination of peptides and proteins. The use of on-line solid phase extraction (SPE) for the preconcentration and sample clean-up prior to a LC separation might be advantageous in peptide analysis [27, 28], however these systems are not included in this review. SPE is only mentioned in this review if it is applied as an interface between two separation systems. A review about preconcentration techniques coupled on-line and in-line to CE was reported earlier [29] and will also not be discussed in this review as well. The majority of the multidimensional systems for proteins and peptides has been applied in the field of proteomics for profiling including a MS detection. Some systems have been applied for quantitation of some peptides or proteins in biological samples. This review will discuss the differences between these two types of approaches. Emphasis will be placed on the design of the systems, whereas their applicability will be

demonstrated with some typical examples. A coupled system can be composed of various separation techniques. Especially LC and CE are widely used. Gas chromatographic methods are not discussed in this paper, because of the decomposition of peptides and proteins at high temperatures. As some recent reviews on 2D-LC systems for proteins and peptides are available [10, 30, 31, in this review special attention will be paid to CE containing systems]. The first section will discuss on-line LC-LC, the second one will overview on-line LC-CE and the third section will summarize on-line CE-CE. The role of MS detection for an increase of selectivity and for providing structure information will also be shown.

2. LC-LC systems

2.1. General aspects

Because LC can be employed in various separation modes, for example normal-phase (NP), reversed-phase (RPLC), size exclusion (SEC), ion exchange (IEX) or affinity chromatography, it is a very powerful tool and offers many possibilities for multidimensional systems. The difference in operation between an on-line coupled column system and a conventional one-dimensional chromatographic system is the direct transfer of analytes from the first column to the second column. This transfer may be the source of three types of problems:

-(1) Solvent incompatibility between the mobile phases of the first and the second system.

-(2) Excessive band broadening between columns during passage of valves, loops or detector.

-(3), The need of a much faster separation in the second dimension compared to the first separation, if a comprehensive system is chosen.

According to (1), the transfer solvent from the first column must allow the focusing of the analytes on the second column and must be miscible with the solvent used in the second dimension. According to (2), the second column preferably must have focusing. This is the reason that, for instance, a SEC-RPLC coupling is very popular. The mobile phases are miscible and the RPLC allows preconcentration at the top of the column of the aqueous SEC fractions. Coupling of LC with CE is complicated because there is generally a large difference in peak volume of the LC and the injection volume of the CE. However, according to (3), the comprehensive mode of a LC-LC system often is more difficult to develop compared to a LC-CE or CE-CE system, because, in general, CE can be performed more easily in a fast mode.

The interfacing in multidimensional LC systems can be divided into two classes: trapping via a loop or direct introduction on the top of the column of the second dimension, both in combination with one or more valves. Trapping in a loop is always possible, while trapping on the column has

limitations. Trapping with an additional trapping column between the two separation columns is also possible. The possibility of trapping on a column depends on the choice of the second retention mechanism and the mobile phase of the first column. A loop has the disadvantage of a possible additional peak broadening.

Most separation modes can easily be combined if their mobile phases are compatible. However, combination is not always possible. The interfacing of NP and RP systems is particularly difficult, due to mobile phase immiscibilities. To overcome this problem, Sonnefeld et al.[32] used a system in which the fraction of interest was transferred from the first (NP) column to a packed precolumn and the NP eluent was removed by passage of an inert gas and vacuum. Once the solvent was removed, the analytes were desorbed from the precolumn using a RP eluent and transferred to the second (RP) column. Another method to overcome solvent inmiscibilities has been developed by Takeuchi et al. [33]. They used a micro column (0.35 mm I.D.) in the first dimension and a conventionally sized column (4.6 mm I.D.) in the second dimension. Due to the reduced peak volume generated by the use of the micro column, solvent removal was not necessary. The resulting 2D separation system was evaluated by the separation of several aromatic hydrocarbons in a fuel for body warmers. Consequently, the use of micro columns in multidimensional separations has allowed coupling of seemingly incompatible separation modes [32, 33]. Micro columns are also better suited than conventionally sized columns for analysis of small sample volumes. Other advantages of miniaturization of a LC system are the low consumption of mobile phases, increased mass sensitivity, higher separation efficiency and the more efficient combination with a MS detector.

A summary of 2D-LC systems is shown in Table I. Some aspects are important: the target proteins and/or peptides, the type of first and second dimension, the type of interface (valves with a loop or valves with a column), the type of detection and whether it is qualitative or (semi-) quantitative analysis. These aspects and some particular remarks are included in Table I.

Table I An overview of 2D-LC systems for proteins and peptides

Compounds	**First dimension**	**Second dimension**	**Interface**	**Detection**	**Remarks**	**Reference**
Standard serum proteins	IEC	SEC	Eight-port valve with two loops	UV	Comprehensive Semi-quantitative Time of analysis: 3 hours	9
Small peptides from porcine adrenal gland	IEC	RPLC	Two valves with trapping on RPLC	LIF	Comprehensive Semi-quantitative Time of analysis: 32 hours Number of spots: 150 Effluent from the IEC is concentrated, quantity of a single resolved peptide is around 16 pmol	83
Proteins from E. coli lysate	IEC	RPLC	Eight-port valve	UV and on-line MS (electro spray)	Comprehensive Semi-quantitative Total peak capacity of over 2500 Sensitivity of around 3 pmol	24
Digest from hunan lung fibroblasts	IEC	RPLC	Two six-port valves with trapping on RPLC	On-line MS	Comprehensive Semi-quantitative	84
Proteins from E. coli lysate	IEC	RPLC	Eight-port valve with trapping on RPLC	Off-line MS	Comprehensive Semi-quantitative	85, 86, 87

Table I, continued

Compounds	**First dimension**	**Second dimension**	**Interface**	**Detection**	**Remarks**	**Reference**
Digested proteins from *S. cerevisiae*	IEC	RPLC	MudPIT technology	On-line MS	Comprehensive Semi-quantitative Time of analysis: 24 hours Number of spots: 23000 (theoretically) The IEC/RPLC combination is not multidimensional, the MS addition is	39
In vivo derived amyloid polypeptides	SEC	RPLC	Six-port valve with trapping on RPLC	On-line MS	Heart-cut Semi-quantitative with known recoveries Time of analysis: 30 min.	13
Peptides from ovalbumine and serum albumin	SEC	RPLC	Four-port valve with two trapping columns	On-line MS	Comprehensive Semi-quantitative Time of analysis: 2.5 hours Number of spots: 50	26
Tryptic digest of bovine serum albumin	SEC	RPLC	Two four-port valves with two trapping columns	On-line MS	Comprehensive Semi-quantitative Time of analysis: 5 hours Number of spots: 520	88
Enkephalins in cerebro-spinal fluid	SEC	RPLC	Two six-port valves with two loops	UV	Heart-cut Quantitative Time of analysis: 30 min. CLOQ: 2 μg/mL	14, 15
Tyrosine-kinase, β-lactamase	SEC	RPLC	Two four-port valves with two trapping columns	UV Off line MS	Comprehensive Semi-quantitative Time of analysis: 2.5 hours Number of spots: 800 (theoretically)	89

Table I, continued

Compounds	First dimension	Second dimension	Interface	Detection	Remarks	Reference
Enkephalins in plasma	SEC, IEC (strong cation exchange) and RP-SPE as sample clean-up	RPLC	Three six-port valves	UV On-line MS	Heart-cut Quantitative Time of analysis: <1hour LOD: 200 nmol/L (UV), 10 nmol/L (MS)	16
Heparine	SEC	IEC	Six-port valve with a loop	Refraction , conductivity	Heart-cut Quantitative Time of analysis: 1 hour LOD < 100 ng	17
Model protein mixtures, digested and labeled	IEC (strong cation exchange)	RPLC	Ten-port valve	MS Acid-labile isotope-coded extractants (ALICE)	Comprehensive Quantitative	40
Human lung fibroblasts	IEC	RPLC	Four ten-port valve with one loop and four trapping columns	UV with off-line MS	Comprehensive Semi-quantitative Time of analysis: 96 min. Number of spots: 1000	37
Digested milk proteins	Affinity chromatogra-phy	RPLC	Three ten-port valves with sample loops	On-line MS	Comprehensive Semi-quantitative	90

2.2. Typical examples of 2D LC systems

A particular example of the analytical power of a 2D system for intact proteins is the coupling of IEC with RPLC, developed by Opiteck et al. for the separation of proteins in an *Escherichia coli* cell lysate [34]. This is the first comprehensive LC/LC system utilizing on-line mass spectrometry for detection, which adds, in essence, a third dimension to this 2D system, because the mass spectrometer can identify the presence of coeluting peaks when they are not resolved by chromatography. The two LC systems are coupled by an eight-port valve equipped with two storage loops which is under computer control. The RPLC column samples the first IEC dimension two or three times per peak. The RPLC effluent is sampled by both a UV detector and an electrospray mass spectrometer. In this way, complex mixtures of large biomolecules can be rapidly separated, desalted, and analyzed for molecular weight in less than 2 h. The 2D chromatogram of *Escherichia coli* lysate is shown in Figure 1.1. This figure clearly demonstrates that the IEC dimension only (See the horizontal line at 37.5 min.) does not have sufficient separation power. The extracted UV absorption data from RPLC and the corresponding total ion current traces from the MS at 37.5 min are presented in Figure 1.2. This figure demonstrates that even the separation power of the 2D system is not sufficient. However, the MS provides additional separation power. The peak at 110 s in the RPLC run (fig. 1.2a) has the mass spectrum shown in Figure 1.3a, with its reconstructed molecular weight shown as 40702 in Figure 1.3b. A search of the Swiss-Prot database shows 38 proteins from *E. coli* within 2% of this molecular weight but only two proteins within 0.2%, GCPE protein (P27433) and hydrogenase-1 small-chain precursor (P19928). The total number of peaks counted in the chromatogram of Fig. 1.2 is roughly 2500. The mass spectrometer can be presented with as little as 3.2 pmol of analyte and still obtain an accurate molecular weight [34]. This example demonstrates the resolving power of the system and is a typical example of peptide mapping with a sensitive on-line MS detection. Most on-line MS detections are based on nanoelectrospray–MS/MS. MALDI-MS/MS is relatively sensitive and also important in the field of proteomics [35], but it can not be coupled on-line to LC. Using off-line MALDI-MS/MS or on-line nanoelectrospray–MS/MS a retrieved peptide mass fingerprint (PMF) is generally used to screen a protein sequence database. A match between the PMF and the patterns computed from the sequence database, employing rules for digest chemistry, are scored and used for protein identification. The success of such an identification depends on the size of the database and its error rate, the number of the matching peptides and their molecular weight (MW), the mass accuracy and the control over the digest chemistry [35].

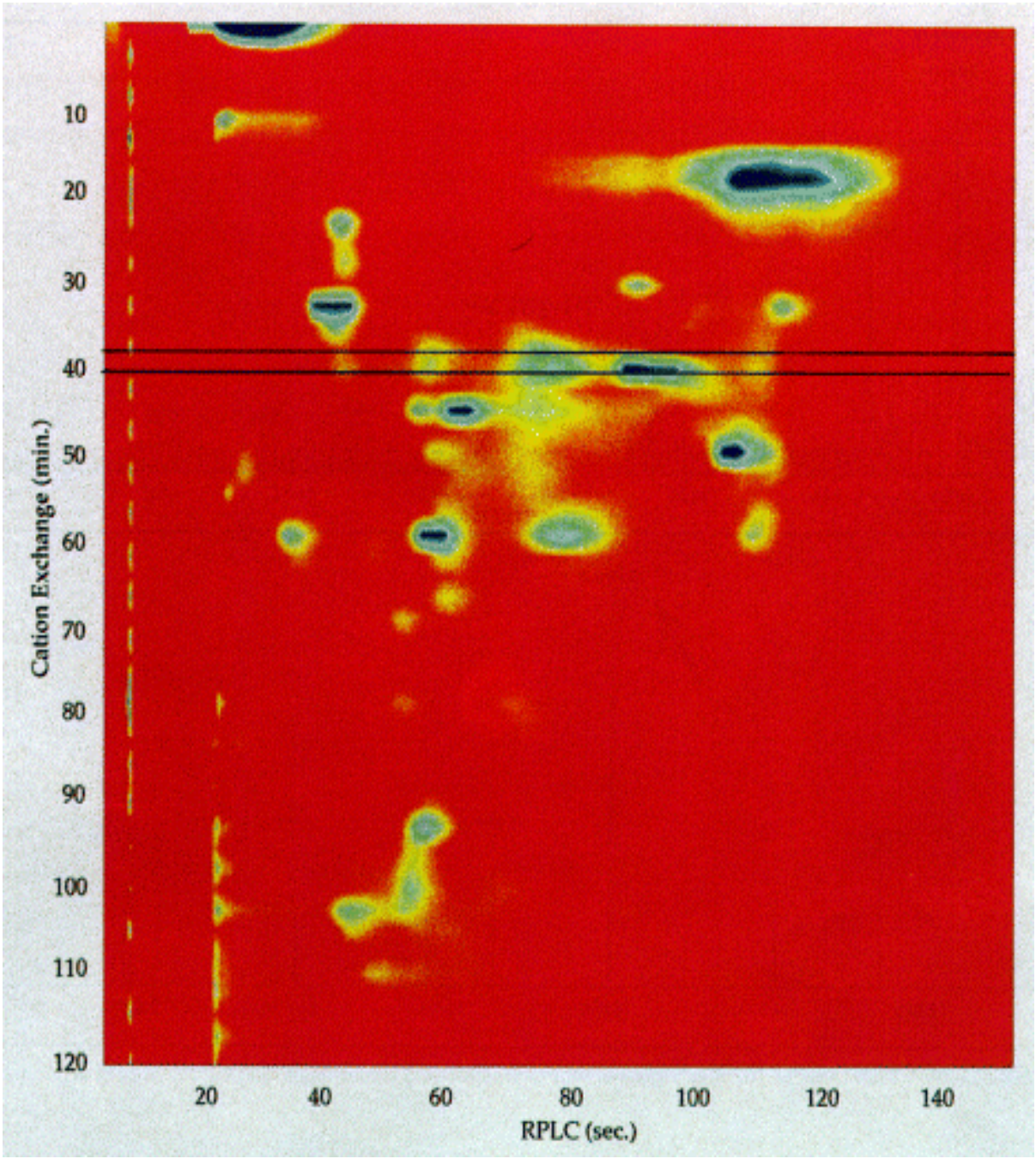

Figure 1.1 2D chromatogram of *Escherichia coli* lysate [34].

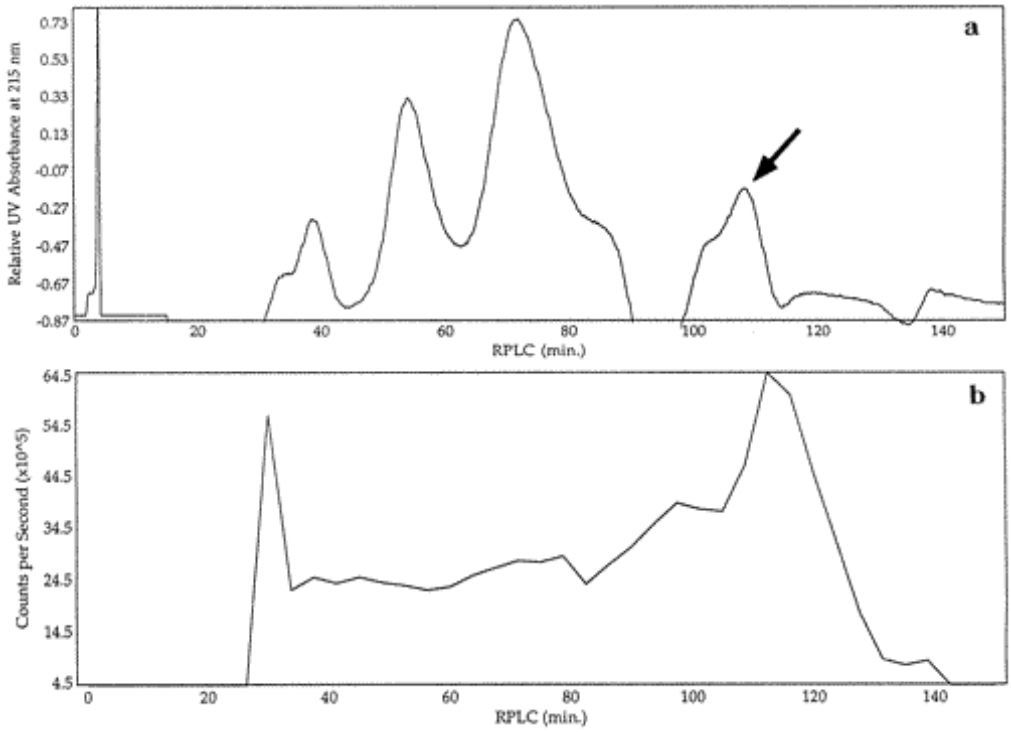

Figure 1.2 (a) UV absorption data from RPLC chromatogram extracted from 37.5 min of Figure 1.1, (b) Corresponding total ion current chromatogram. [34].

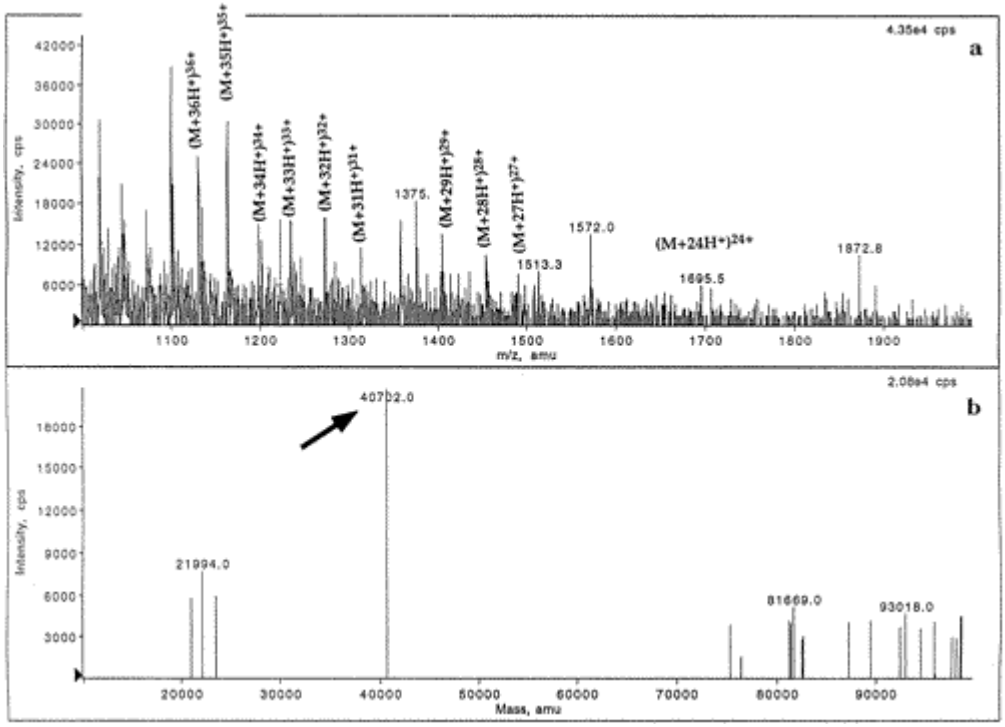

Figure 1.3. (a) Mass spectrum from peak at 110 s of Figure 1.2. (b) Corresponding Hypermass reconstruction of charge envelope [34].

A typical illustration of comprehensive LC-LC is the high resolution 2D-LC system based on high speed RP chromatography [36]. This system is developed for separations of peptides and proteins using non-porous 1.5 µm silica beads and conventional low-void-volume chromatographic equipment to prevent additional peak broadening. The 2D system consists of either an anion or a cation exchanger in the first dimension and two equivalent non-porous 14x4.6 mm i.d. RPLC columns in the second dimension which operate faster compared to other systems described in the literature [34, 26]. While the first RP column is loaded with a fraction of the effluent from the ion exchanger, the analytes on the second column are eluted. Subsequently, after switching the valves, trapping is performed onto the second column while analytes are eluted from the first column. Consequently, the interface is the switching valve combined with trapping capabilities of the RP columns. An improvement of this fast 2D-LC system has recently been developed [37] for the analysis of peptides and small proteins The system reproducibly resolves ~ 1000 peaks within a total analysis time of 96 min. The design of the fully integrated platform for peptide and protein mapping is illustrated in Figure 2.

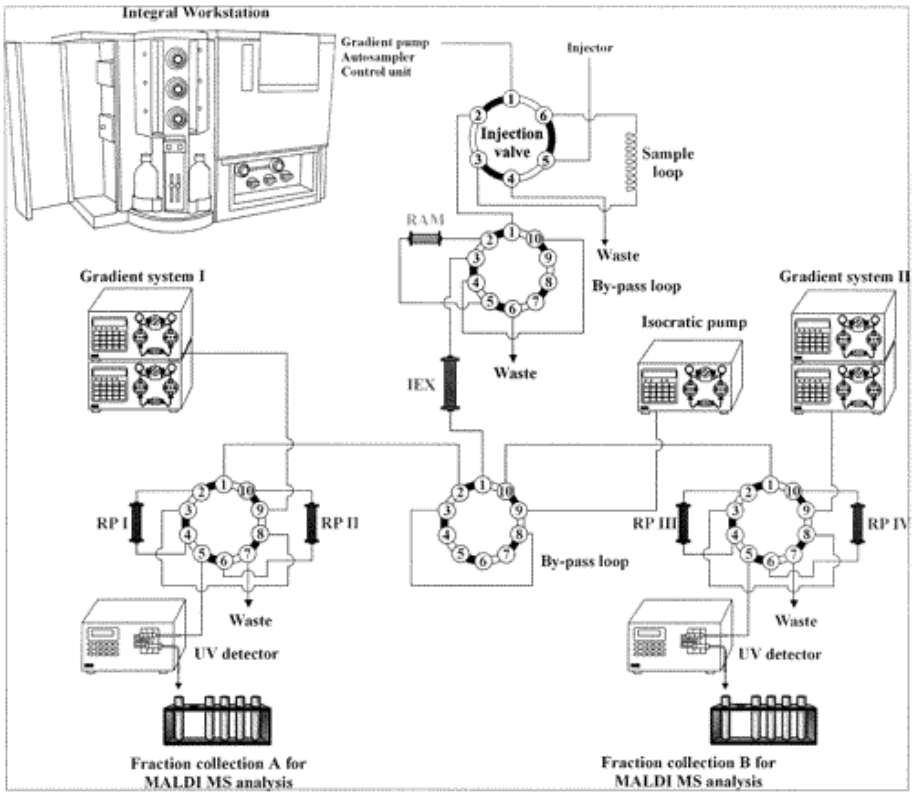

Figure 2. Schematic representation of the on-line comprehensive 2D IEC-RPLC system, including an integrated sample preparation step [37]. The restricted access material is indicated with RAM.

It consists of a sample fractionation column with silica based restricted access materials (RAM) based on the SEC principle coupled on-line with a comprehensive 2D-LC system. Peptides and small proteins are being enriched, whereas higher-molecular-weight matrix components can be flushed directly to waste. This size selective sample fractionation step is followed by anion or cation exchange chromatography as the first dimension. A new column switching technique, including four parallel RP columns, is employed in the second dimension for further separation. The system has been applied to protein mapping of human hemofiltrate as well as cell lysates originating from a human fetal fibroblast cell line. Figure 3.1. shows the separation of human hemofiltrate on the analytical IEC column after first being subjected to selective enrichment on the RAM, while Figure 3.2. shows a couple of reversed-phase chromatograms from selected fractions of the IEC, exemplifying the high resolving power within 8 min of analysis time. The 3D RAM/IEC /reversed-phase separation of human hemofiltrate is shown in figure 3.3. [37]. Selected peaks are collected and analyzed off-line by MALDI-TOF MS. Figure 3.4. is an illustration of this application. It shows a MALDI-TOF spectrum corresponding to the peak (arrow) in the chromatogram, which originates from a human hemofiltrate sample. In this case, two masses were observed from the single UV peak [37]. On-line MS detection might be possible if other modes of MS detection, e.g. electrospray MS, are used.

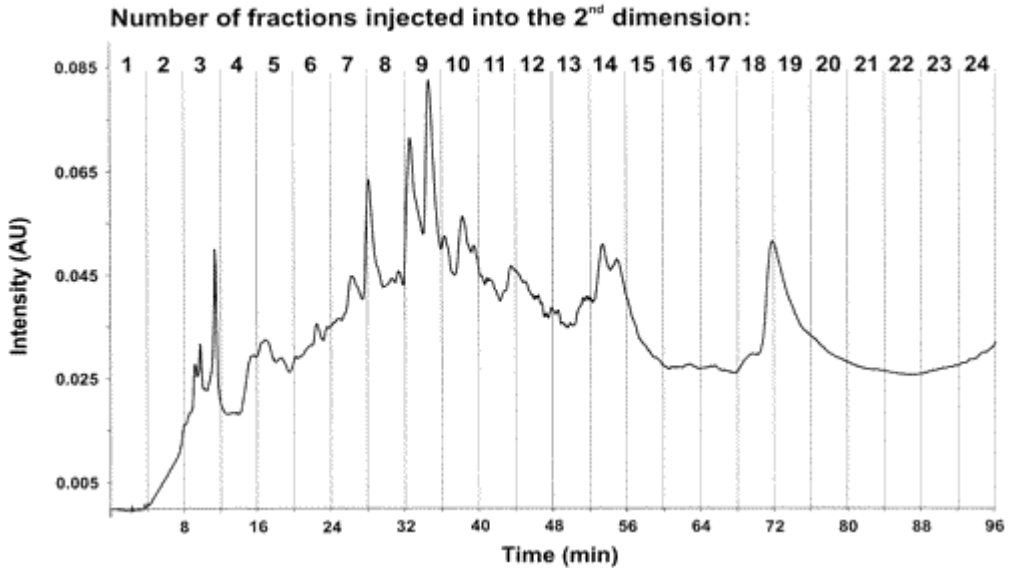

Figure 3.1. The separation of human hemofiltrate on the analytical IEC column in the first dimension after first being subjected to selective enrichment on a RAM. Fractions (24 in total) were continuously transferred to the second dimension in 4-min intervals for subsequent analysis by reversed-phase chromatography [37].

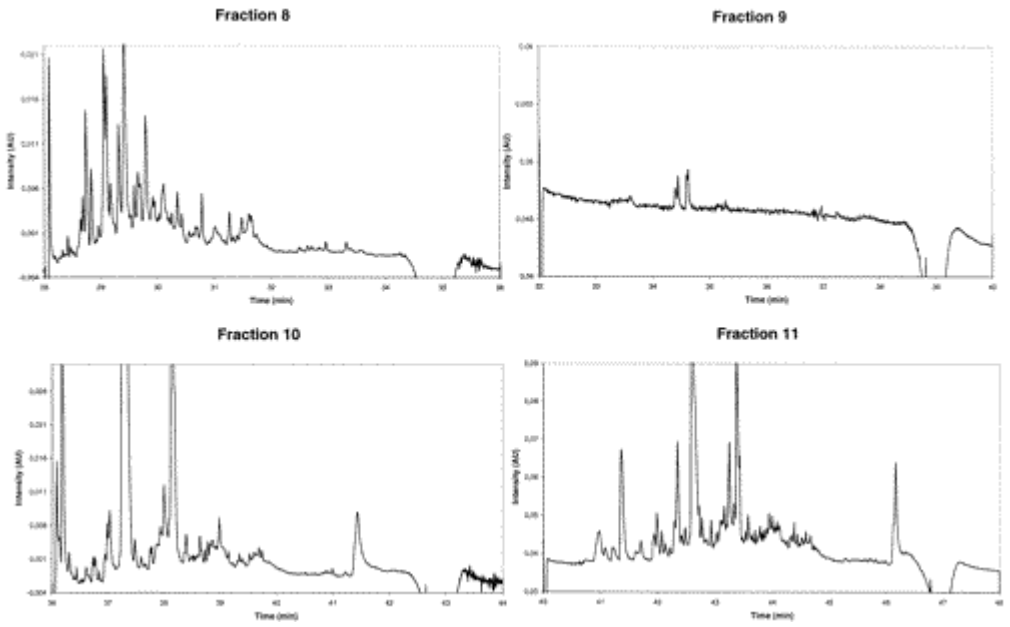

Figure 3.2. Selected reversed-phase chromatograms corresponding to a complete two-dimensional RAM/IEC /reversed-phase run exemplifying the high resolving power within 8 min of analysis time [37]. The fraction number mentioned on the cation exchange chromatogram corresponds to the fraction indication on the reversed-phase chromatograms in Figure 3.1.

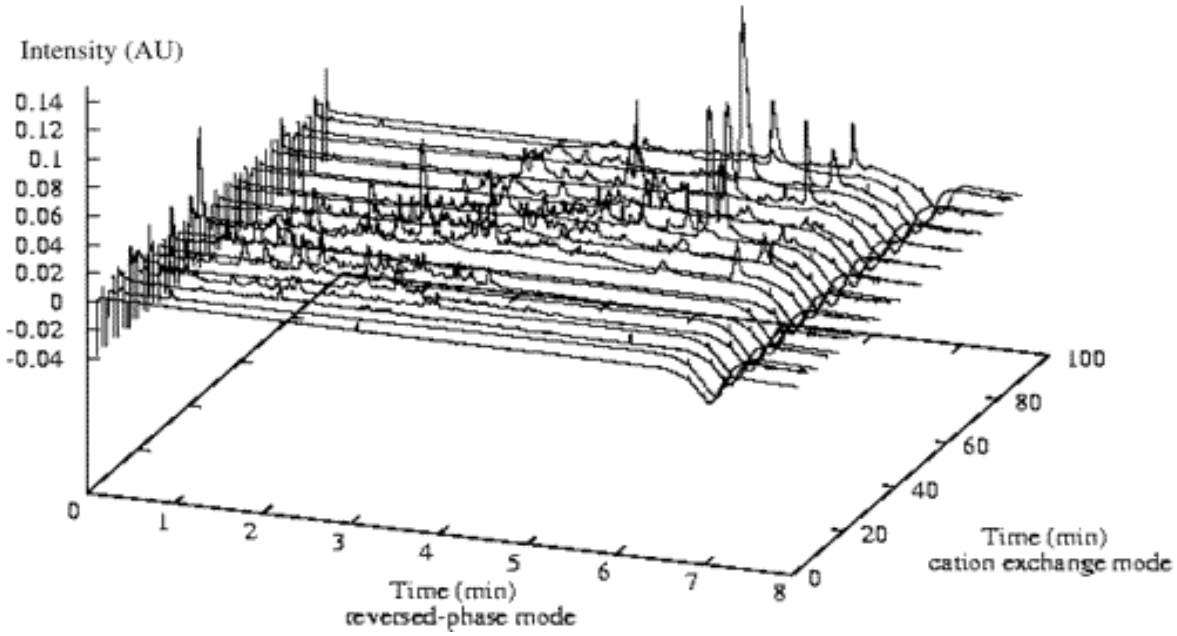

Figure 3.3. 3D RAM/IEC /reversed-phase separation of human hemofiltrate [37].

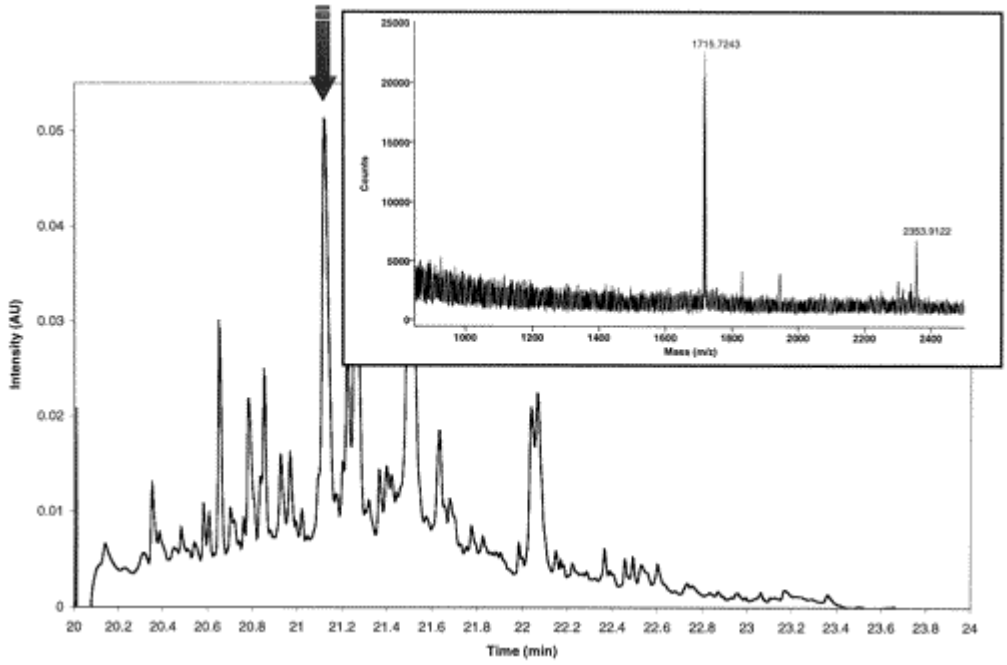

Figure 3.4. Typical MALDI-TOF spectrum corresponding to the peak (arrow) in the chromatogram, which originates from human hemofiltrate sample. In this case, two masses were observed from the single UV peak [37].

The previously discussed separation systems are on-line. Vissers et al. [38] developed an automated at-line 2D capillary LC method which is based on micro fractionating, automated reinjection, and rechromatography using an automated micro column switching set-up to separate complex peptide mixtures from different origins. Although this procedure is not on-line, it is advantageous that the parts necessary for the sample transfer from the first dimension to the fraction collector are commercially available. The first-dimension separations were carried out by either RPLC or strong anion exchange chromatography. The second dimension separation was in all cases RPLC. Because of the large robustness of this system it can possibly be applied for the quantitation of peptides or proteins as well.

Another particular example is the powerful and well-known system described by Wolters et al. [39]. It is an automated method for so-called shotgun proteomics, named multidimensional protein identification technology (MudPIT). MudPIT works fast and is focused on the identification of proteins and peptides. The MudPIT-system itself integrates a strong cation exchange resin (SCX)

and RP resin in one column. The RP material is positioned directly after the SCX material. Consequently, the combination of the SCX and the RP resin can be imagined as two different on-line coupled columns without an interface between them. After loading the peptides onto the SCX, the MudPIT experiment consists of a couple of identical cycles. Each cycle integrates several steps:, equilibrating with buffer of a specific pH, elution of a peptide fraction from the SCX column onto the RP column using a salt gradient and subsequent elution with a RP gradient to a mass spectrometer. Depending on the pH, peptides with a particular pI elute off the SCX and are further separated on the RP material in relation to their hydrophobicities. Consequently, multiple 1D chromatograms are retrieved. The theoretical peak capacity of the total MudPIT system is ~23000 [39]. MudPIT is a powerful tool for proteomics. The system is capable of sensitive detection of peptides, due to the at-line concentration procedure prior to peptide loading onto the SCX and the MS detection, however it has not been developed for quantitative analysis. It can be questioned whether this integrated combination of SCX/RP is really multidimensional. Using the described set-up the whole effluent from the SCX dimension is directly transported to the RP dimension.

From Table I some conclusions can be drawn. The analytes of interest are often peptides retrieved from digested proteins. In case of the digested proteins, off-line sample handling is performed before injection onto the coupled system. This introduces at one hand the possibility of analyte concentration, at the other hand it is time consuming. Most of the systems use RPLC for the second dimension. This has two possible advantages: concentration on top of the RP column and the use of a loop with additional band-broadening is not necessary. In the systems which do not use RPLC in the second dimension, a loop is employed for interfacing. Another aspect is the low number of heart-cut systems, while all the described heart-cut systems have been validated for performing quantitation of proteins or peptides, contrarily to the comprehensive systems. Two examples of mapping systems are reported which have also been validated for quantitations of peptides [40, 41].

3. LC-CE systems

3.1. General aspects

LC-CE coupling instead of LC-LC combination is generally more orthogonal. Also, the high separation speed of CE is advantageous, especially in the development of comprehensive multidimensional systems. In addition CE has a large separation efficiency, however, especially when trace-level concentrations have to be determined, the analyte detect ability in units of concentration is often insufficient.

Next to the interfacing problems discussed in the section on LC-LC, the on-line coupling of LC and CE may be the source of two additional problems: The large difference in peak volume of the LC and the injection volume of the CE and the way the electrode is used at the coupling side of the CE and the voltage is applied.

The transfer solvent from the first dimension preferably must allow stacking of the peptides or proteins in the CE capillary. Consequently, the ionic strength of the LC effluent has to be as low as possible. While most LC-CE systems are comprehensive because of the use of a slowly eluting LC system, fast CE (FCE) [42, 43] can be necessary to enable comprehensiveness without this requirement for the LC system, although normal CE is often relatively fast in contrast to LC. It is important for the CE injection system to sample the first column as frequent as possible.

The differences in peak volumes of the LC (μL range) and the injection volume of the CE (nL range) are large in LC-CE coupling. Generally a T-piece can reduce these differences. However in this case, only a part of the effluent from the LC will be injected into the CE. The major part is transported via the T-piece to waste. If this approach is chosen, focusing in the CE capillary is even more important to diminish loss in sensitivity. Another solution may be the use of a nano LC dimension with effluent peaks comparable to the injection volume of the subsequent CE dimension [44]. However, a disadvantage of using nano LC in the first dimension is the relatively small amount of sample that can be injected. Consequently, using nano LC, the sensitivity of the total system will not always be improved.

The stainless steel tubing of the LC dimension in most cases serves as the inlet electrode for the CE [9, 11, 12, 37, 43, 44, 45, 46]. In this case, the inlet electrode of the CE is grounded, while the outlet electrode has a positive or a negative potential [9, 11, 12, 37, 43, 44, 45, 46]. This is simple, however, it complicates the addition of on-line MS detection compared to a LC-LC system. However, in principle the positioning of a separate electrode is also possible.

In Table II an overview is given of the available multidimensional LC-CE systems. Some aspects are important: the type and dimension of the LC dimension, the speed of the CE dimension, whether the coupling is heart-cut or comprehensive, the type of interface, the detection mode and whether the system has been developed to quantify peptides or proteins or is more suitable for profiling purposes.

Table II An overview of LC-CE systems for peptides and proteins.

Compounds	**First dimension**	**Second dimension**	**Interface**	**Detection**	**Remarks**	**Reference**
Tryptic digests of ovalbumin, fluorescently labelled	RPLC	CZE	Two six-port valves with two loops	Fluorescence	Comprehensive Semi-quantitative Time of analysis: 3 hours Number of spots: 420 (theoretically)	48
Proteins and peptide fragments of horse heart cytochrome C and bovine heart cytochrome C	RPLC	CZE	Six-port valve	Fluorescence	Comprehensive Semi-quantitative Higher electric field strengths might be possible Influence of hydrodynamic flow reduced by using 15 μm i.d. capillaries	49
Tryptic peptides of horse heart cytochrome C	RPLC	CZE	Eight-port valve	LIF	Comprehensive Semi-quantitative	42
Tryptic peptides of horse heart cytochrome C	RPLC	FCE	Optical gating injection system	Fluorescence	Comprehensive Semi-quantitative Effective CZE capillary: 1.2 cm	42
Peptides of methoxy-arginine horse heart cytochrome C and trypsin	RPLC	FCE	Optical gating injection system	Fluorescence	Comprehensive Semi-quantitative Time of analysis: 7 min. Number of spots: 25 Separation length CZE capillary: 2 cm	43

Table II, continued

Compounds	First dimension	Second dimension	Interface	Detection	Remarks	Reference
Standard proteins thyroglobulin, bovine serum albumin, chicken egg albumin, horse heart myoglobulin	μSEC	CZE	Four-port valve	UV	Comprehensive Semi-quantitative SEC flow rate 180 nL/min	42
Proteins (human, horse)	μSEC	CZE	Six-port valve with T-piece	UV	Comprehensive Semi-quantitative Time of analysis: 2 hours SEC flow rate 360 nL/min	45
Standard proteins thyroglobulin, bovine serum albumin, chicken egg albumin, horse heart myoglobulin	μSEC	CZE	Optical gating injection system	UV	Comprehensive Semi-quantitative Time of analysis: 2 hours Two SEC columns: 100 and 250 μm i.d. SEC flow rate is 235 nL/min (column dimension: 250 μm i.d.) and 20 nL/min (column dimension: 100 μm i.d.)	44

Table II, continued

Compounds	First dimension	Second dimension	Interface	Detection	Remarks	Reference
Proteins and amino acid standards in urine	μRPLC	CZE	Optical gating injection system	LIF	Comprehensive Semi-quantitative Time of analysis: 1 hour Number of spots: 400 Direct observation and routine manipulation of the micro-HPLC and CZE capillaries	46
Enkephalins in cerebro-spinal fluid	SEC	CZE	Various valves, transvers flow and T-piece	UV	Heart-cut Quantitative LOQ: 2.5 μg/mL Positioning of a trapping column between the SEC and the CZE	11, 12
Bradykinin, neurotensin , lecine enkephalin	RPLC	CZE	Transverse buffer flow	UV, MS	Semi-quantitative RPLC-CZE coupling comprehensive Time of analysis: 15 min.	91
Bovine albumine, myoglobin	Gel filtration	CIEF	Eight-port valve with internal sample loop and dialysis membrane	UV	Comprehensive Semi-quantitative Time of analysis: 30 min. Desalting with a dialysis membrane after the gel filtration column	92
Proteins in several cell lysates	CGE	RPLC	Eight-port valve with two loops	UV	Comprehensive Semi-quantitative Time of analysis: 2 hours	50
Peptides from the salivary glands	CIEF	RPLC	A valve with a loop as microinjector	UV	Comprehensive Semi-quantitative	51

3.2. Typical examples of LC-CE systems

The combination of very short capillaries with normal high voltages (5-25kV) will give very fast CE analyses [37, 47]. However, if the speed of these analyses becomes very fast, a fast injection method will be necessary as well. In this case a valve with a loop can not be used for the coupling. A unique optical-gating injection and detection system is developed by Larmann et al [42] to enable injections that are fast in comparison with the CE analysis time. This system is based on a laser. The samples to be analyzed are tagged with fluorescein isothiocyanate (FITC), thus becoming sensitive for the 488 nm laser. The initial laser beam is split into a gating beam, focused near the injection end of the capillary and containing 95% of the laser power, and a probe beam, focused near the exit end of the capillary and containing the remaining 5% of the laser power. The optical-gating injection can be considered as an "inverse" injection method. As long as the gating beam is focused on the capillary, the analyte in the sample passing through the beam is photodegraded by the intense light of the gating beam. To inject a plug, the gating beam is temporarily blocked (usually for 5-50 ms) with a computer-controlled shutter. This allows the passage of a small plug of unaffected material through the capillary between the beams, where the components of the plug are separated and detected by their fluorescence when they pass the probe beam. No interface valve is needed because CE injections are controlled by the gating beam. The FCE system enables complete CE analysis in as little as 3 s. Because of the high speed, there is more freedom in monitoring the first separation dimension. Figure 4 is a schematic diagram of the capillary mound of the FCE instrument. The capillary is only 10 μm in internal diameter (i.d.) and a low-concentration CE buffer (10 mM) is used. The effective separation capillary (distance between the gating beam and probe beam) is 1.2 cm. Consequently, with such a short capillary, the CE efficiency is limited [43].

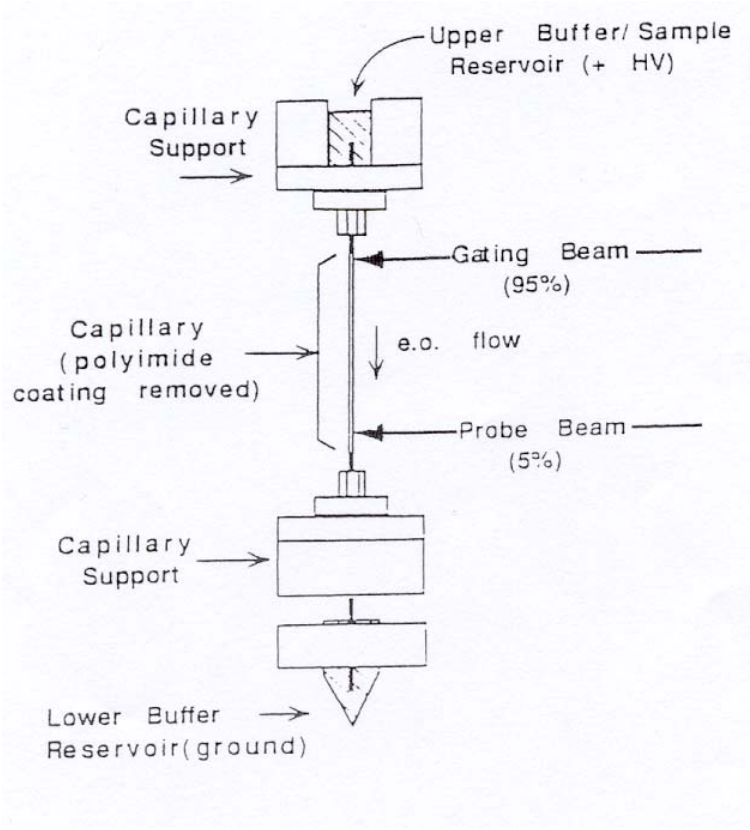

Figure 4. Diagram of the capillary mount of the fCE instrument [42].

One possible application of this method is the rapid fingerprinting of proteins: tryptic digests of known and unknown proteins can be fluorescently tagged and analyzed semi-quantitatively by this method [42]. An application of FCE with another injection system is discussed and shown later.

In the development of the comprehensive coupling of LC with CE, Bushey and Jorgenson initially used conventional LC columns and a sample loop as interface for the analysis of peptides samples [48, 49]. The use of LC micro columns in a comprehensive system is attractive, as mentioned earlier. However, the use of micro columns with small eluent volumes makes the collection of sample in loops impractical because of peak broadening. The coupling of separation techniques that operate with low flow rates requires the use of an interface design capable of transferring small volumes from the LC to the CE, while minimizing extra peak broadening. Therefore, an interface was designed, allowing micro-LC and CE to be combined in a comprehensive 2D system [44]. Figure 5 represents the instrumental set-up. In the interface a cross flow of buffer has been used. The cross flow is the buffer flow from the CE buffer

reservoir via the "flush buffer in" to the waste via "flush buffer out" as indicated in Figure 5. The cross flow controls the injections of eluted LC mobile phase onto the CE capillary and the system is called the Transverse Flow Gating Interface.

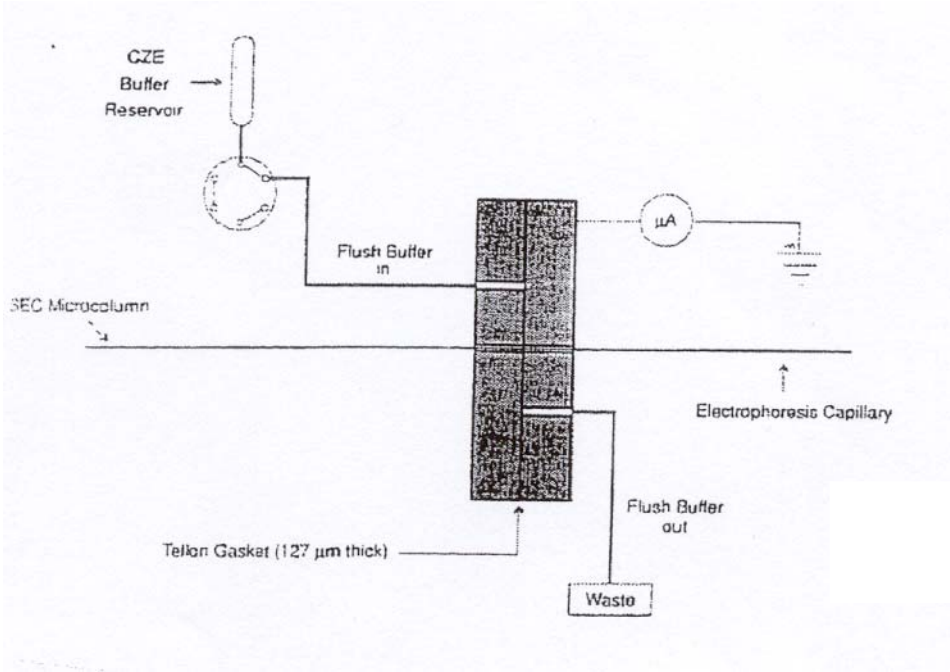

Figure 5. Schematic of instrumental setup for 2D SEC-CE using a Transverse Flow Gating Interface. [44]

Since the position of the capillaries with respect to each other in the interface is critical for a successful sample transfer from LC to CE, it was necessary to construct the interface from a transparent material (polycarbonate polymer, Lexan) [44]. The chemical resistance to acids, bases, and certain organic solvents of Lexan makes it an attractive material. Problems, such as air bubbles, trapped between the two capillaries, can easily be diagnosed with the design (Transparent Flow Gating Interface). The design also allows the positioning of the fritted outlet of the LC micro column directly in the interface and in the transverse buffer flow. This interface offers a large amount of flexibility in its operation. Adjustable parameters include the distance between the capillaries, the transverse buffer flow rate, injection time and voltage. Figure 6 gives an indication of the resolving power of such a system. Moreover, it is an illustration of FCE as well. A SEC-CE system, using an interface with T-piece, a flush stream of buffer and also based on a type of flow gating, has recently been developed [11, 12].

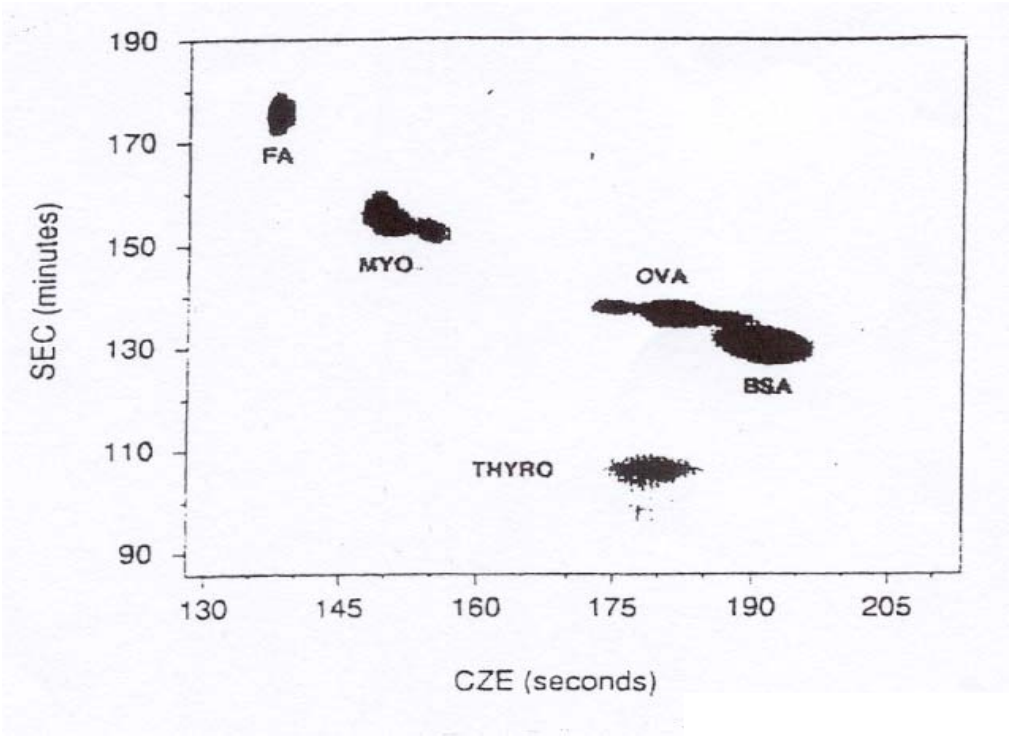

Figure 6. Separation of protein standards by 2D SEC-CE with the flow gating interface and a 250 μm i.d. micro column. Each protein was present at 0.5% (w/v) with 2.5% (w/v) formamide. FA: formamide MYO: horse heart myoglobulin OVA: chicken egg albumin BSO: bovine serum albumin THYRO: thyroglobulin [44].

The flush stream is not positioned across, but it is applied in the interface using a switching valve. The SEC column is of normal size (4.6× 30mm), however, a preconcentration column is positioned between the SEC and the CE for trapping of a SEC fraction. Although this system is not comprehensive, a group of structurally related enkephalins could be quantified with a LOQ of 20 ng in cerebrospinal fluid.

Beside the most important LC-CE couplings, there are two CE-LC system included in Table II. These are a relatively old gel electrophoresis-liquid chromatographic system developed by Rose et al [50] and a relatively new CIEF-RPLC design [51]. The system of Rose was not further employed because it has one large disadvantage: the use of the polyacrylamide gel columns was not very reproducible, due to degradation of the polyacrylamide matrix with time and irreproducibility of the polymerization reaction. The advantage of CIEF in the first dimension of the system of Chen et al. [51] is its large preparative capability compared to most of the capillary-based electrokinetic separation techniques since the entire capillary is initially filled with a solution containing proteins/peptides and carrier ampholytes for the creation of a pH gradient in the capillary. The focused peptide fractions, which have a similar pI, are coinjected into the second RPLC dimension and further resolved by their differences in hydrophobicity [51].

From Table II, a number of general conclusions emerge. Using LC-CE, the analytes of interest are peptides from digested proteins as well as intact proteins. Many systems are developed for

detection of proteins. Most of the systems use RPLC or SEC as the first dimension. The eluent of RPLC allows stacking in the CE system because it contains relatively small amounts of ions. The effluent of SEC can allow stacking if only small amounts of salts are used in the mobile phase of the SEC. The transfer of all analytes in an effluent plug from the LC dimension into the CE capillary is for all systems impossible. Always a fraction of the analytes in an effluent plug is injected into the CE. Only a few heart-cut systems for quantitation of proteins or peptides have been described. The comprehensive systems have not been developed for the quantitation of proteins or peptides, however quantitative analysis may be possible.

4. CE-CE systems

4.1. General aspects

Electrophoretic separations can be based on various mechanisms: charge to mass ratio (capillary zone electrophoresis: CZE, isotachophoresis: ITP) isoelectric point (capillary isoelectric focusing: CIEF), with size (capillary gel electrophoresis: CGE) and even differences in hydrophobicity (micellar electrokinetic chromatography: MEKC). Consequently, when combining different electrophoretic principles for the development of a CE-CE system it is possible to develop orthogonal 2D systems. Compared to LC-CE combinations a CE-CE coupling may be easier to realize: the effluent peak of the first capillary will generally have a volume similar to the injection amount on the second capillary, however the transfer of this small volume may be difficult. Another advantage is the remarkable separation power [52, 53].

Additional problems in CE-CE coupling compared to LC-CE couplings are: The samples must contain only low concentrations of salt and the low injection amount of the first dimension. Especially for sample components with low concentrations, large sample volumes have to be injected in order to obtain detectable amounts of components. A possibility is concentration the sample of components to obtain an acceptable sensitivity. For this purpose, field amplification [29, 54, 55] or sample stacking [29, 56, 57] is often applied, however, these techniques can result in a disastrous decrease in separation power [58], especially for large sample volumes. Another solution is to improve the detection, by use of detectors which are more sensitive than UV, such as LIF [59, 60], or by altering the detection cell configuration, such as in multireflection detection cells [61], Z-cell and bubble cell [62, 63]. The injection of certain biological samples e.g. urine or cerebro-spinal fluid is generally possible desalting is often needed. Desalting is also necessary to enable stacking.

A summary of the de CE-CE systems is shown in Table III.

Table III An overview of CE-CE systems for peptides and proteins

Compounds	**First dimension**	**Second dimension**	**Interface**	**Detection**	**Remarks**	**Reference**
Angiotensin	ITP	CZE	Counterflow	UV	Comprehensive Semi-quantitative	66
Peptides (tryptic digest from *Aplysia california*	CZE	CGE	Moving capillary	Fluorescence	Comprehensive Semi-quantitative Time of analysis: 30 min. Number of spots: 780 Use of polyacrylamide gel	70
Cytochrome C, ribonuclease A, carbonic anhydrase II	CIEF	ITP	Microdialysis junction	UV	Comprehensive Semi-quantitative Time of analysis: 40 min. Number of spots: 150	93
Bovine carbonic anhydrase, rabbit actin, bovine serum albumin and human hemoglobin	CIEF	CGE	Dialysis interface	UV	Not multidimensional Semi-quantitative	71
Ribonuclease	CIEF	CZE	Dialysis interface	UV	Not multidimensional Semi-quantitative	94

Table III, continued

Compounds	**First dimension**	**Second dimension**	**Interface**	**Detection**	**Remarks**	**Reference**
Proteins extracted from HT29 cancer cells	Submicellar CE	CZE	Resembling the optical gating injection system	LIF	Multiple heart-cut with comprehensive reconstruction Semi-quantitative Time of analysis: 8hour Number of plates: 100000 Sensitivity in the low zepto moles	72
Fluorescently labeled products from tryptic digests of cytochrome c (from bovine heart)	MEKC	CZE	Chip technology	Fluorescence	Comprehensive Semi-quantitative Time of analysis: 15 min. Estimated number of spots: 500-1000	80
Fluorescently labeled products from tryptic digests of β-casein	MEKC	CZE	Chip technology	LIF	Comprehensive Semi-quantitative Time of analysis: 15 min. Estimated number of spots: 150	81

Table III, continued

Compounds	First dimension	Second dimension	Interface	Detection	Remarks	Reference
Fluorescently labeled products from tryptic digests of ovalbumin, human hemoglobin and bovine hemoglobin	MEKC	CZE	Chip technology	LIF	Comprehensive Semi-quantitative Time of analysis: 15 min. Number of spots: 4200	82
Green fluorescent protein, ovalbumin, low-density lipoprotein and trypsin inhibitor	CIEF	CGE	Chip technology with four valves on chip	Fluorescence	Comprehensive Semi-quantitative Time of analysis: < 10 min.	95

4.2. Typical examples of CE-CE systems

4.2.1. Coupling of CITP with CZE

The coupling of capillary ITP (CITP) with CZE in an on-line comprehensive arrangement is a promising technique for combined trace enrichment and analysis of samples with low limits of detection (LOD) [64]. The ability of ITP to introduce sample volumes in the μL range in combination with the 10- to 1000-fold preconcentration factor, compared to nL-size injections in normal CZE [65] is attractive for the analysis of low-concentration analytes in complex matrices. However, although these methods are 2D, in most studies the cITP is only used as a concentration step prior to normal CZE. Recently, one comprehensive cITP-CE system is introduced for the determination of angiotensin [66]. This system uses a second UV-Vis detector which is positioned after the ITP dimension to improve injection timing. The interfacing is performed by using a counterflow in a principle similar to the Transferse Flow Gating Interface.

4.2.2 Coupling of CIEF with CGE

If CIEF is coupled to CGE, a system can be designed based on the same principles as 2D gel electrophoresis. Consequently, it may have be similar separation power. Using CIEF, amphoteric compounds such as proteins are being focused in a pH gradient, established by carrier ampholytes, under a high voltage. A solution of phosphoric acid is usually used as the anolyte and a solution of sodium hydroxide as the catholyte. These solvents both differ from the buffers used in CZE. As a result, it is difficult to combine CIEF with CZE. The presence of high-density gel in a second CE dimension changes the separation mechanism to a size-based separation, minimizes solute diffusion [67-69] and prevents analyte adsorption to the capillary walls [70]. These factors all can improve separation efficiency for the proteins.

One system consisting of CIEF and CGE has been reported [71] for the analysis of hemoglobin (Hb), bovine carbonic anhydrase, rabbit actin and bovine serum albumin. A dialysis interface with a hollow fiber was developed to integrate CIEF with CGE into an on-line system. With the dialysis interface, small molecules can be introduced to or removed from the separation channel to change the ionic strength in the capillary and, consequently, the zeta-potential and the migration of the analytes. This system is easy to operate with only one high voltage source and three electrodes. Figure 7 is a schematic diagram of this 2D-CE/CE system. The focusing is started when a voltage is placed over the CIEF capillary. After the focusing and catholyte buffer replacement, the anode (Figure7) was moved into the anolyte compartment of the second dimension capillary. A hemoglobin (Hb) sample containing various molecular weight markers was separated to check the

effectiveness of this system. Figure 8A shows the CIEF electropherogram of a Hb sample, without CGE separation. The four peaks indicate the four Hb variants A, F, S, and C. They have pI values of 7.10, 7.15, 7.25, and 7.50, respectively. The 1D CGE electropherogram of the Hb sample is shown in Figure 8B, while the 2D separation is shown in Figure 8C. This is not a real multidimensional system, since the CIEF buffer is transported continuously into the CGE. It could have been multidimensional if every fraction with the same pI had been separated individually in the CGE. The elution order of A, F, S and C is the same in the 1D CIEF electropherogram as in the 2D electropherogram, because their size is approximately the same. If this was not the case, their elution order in the CGE could be changed. It should be noted that compounds a, b and c in fig. 8B have not been identified in Figure 8C. The elution order of these substances could not be determined in the 2D system. Consequently in principle, using this set-up the CGE dimension can reverse the resolution achieved by CIEF.

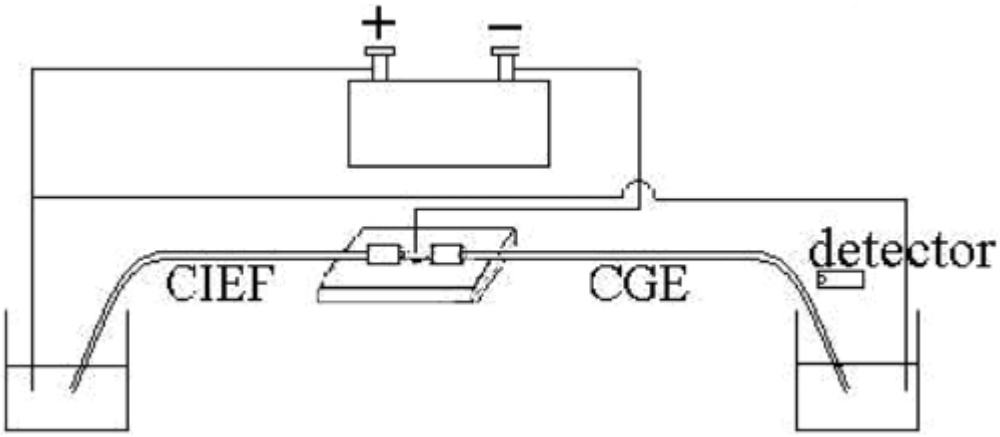

Figure 7. Set-up of a 2D-CIEF/CGE system [71].

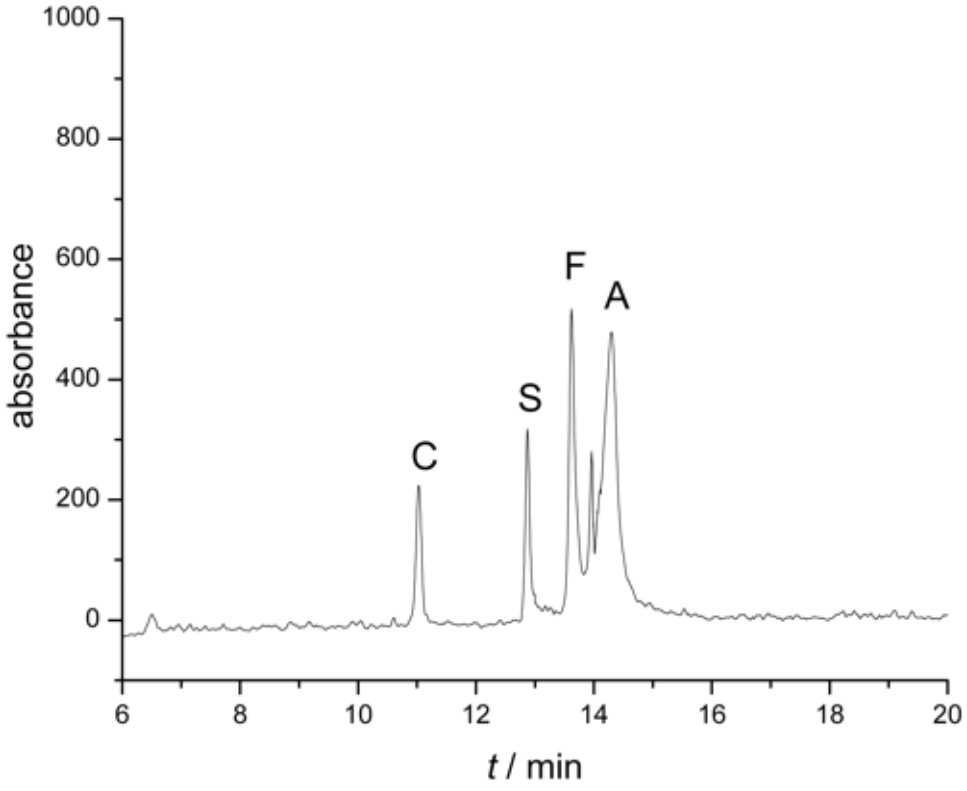

Figure 8A. CIEF of hemoglobin. Hb (0.2% w/v) was dissolved in 50 mM Tris-HCl containing Pharmalyte (2% v/v) and TEMED (0.1% v/v) [71]. A, F, S and C stand for the four variants of hemoglobin with different pI.

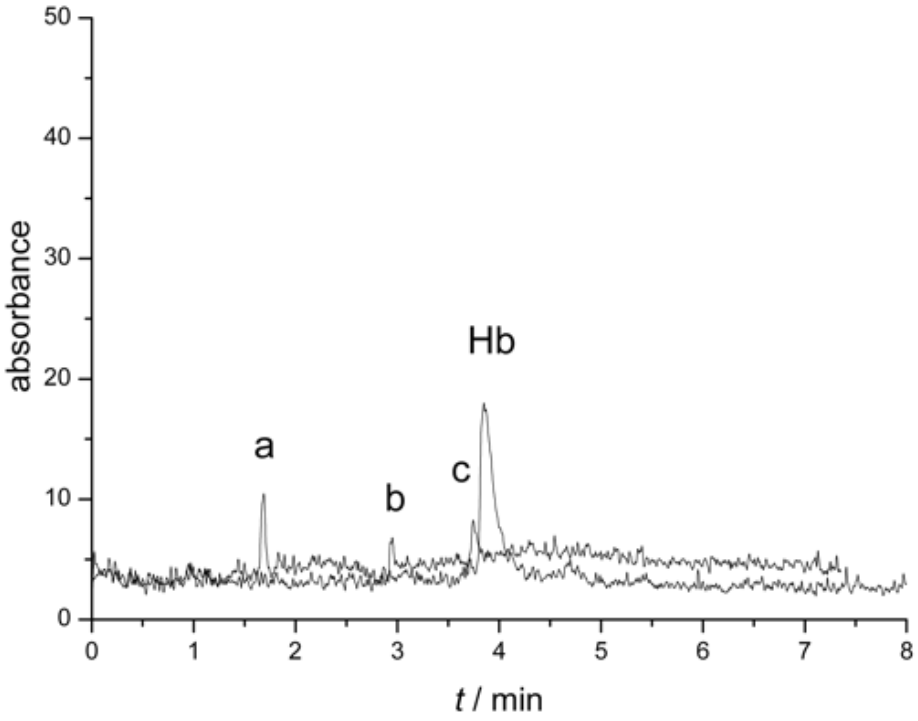

Figure 8B. CGE of proteins: (a) carbonic anhydrase (bovine, 31000), (b) actin (rabbit, 43000), and (c) albumin (bovine serum, 66200) and hemoglobin (Hb), all 0.1% (w/v) in CGE buffer. The concentration of Hb is 0.2% (w/v) SDS was used as both the buffer and the electrolytes [71].

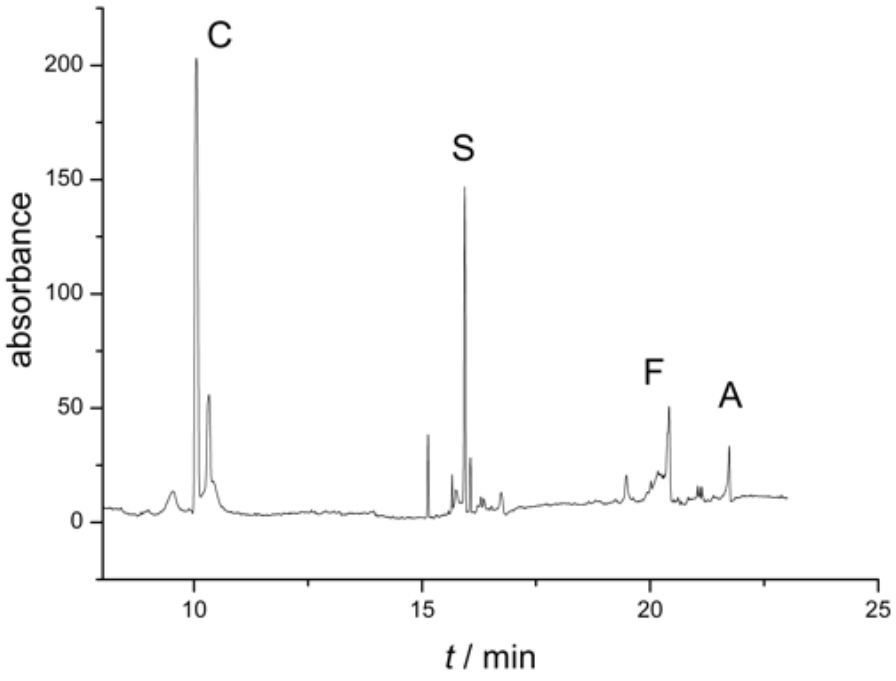

Figure 8C. 2D electropherogram of Hb. A, F, S and C stand for the four variants of hemoglobin with different pI [71].

4.2.3. Coupling of CZE with CGE

As previously stated, in gel electrophoresis analyte migration is hindered by the structure of the high-concentration and cross-linked polyacrylamide gels (sieving and/or interaction with the polymer matrix). One system, based on the combination of CZE and high-density gel electrophoresis. was reported for the analysis of some target peptides, a tryptic digest of trypsinogen and an individual B2 neuron from the marine mollusk *Aplysia californica* [70]. The second dimension consists of a number of channels. These channels are filled with *N,N'*-methylenebis(acrylamide) cross-linked polyacrylamide gel, chemically bound to the inner wall of the glass channel. Reproducible, bubble-free and stable gels are easily obtained in the thin channels. Injection into the second dimension is performed by moving the outlet end of the first capillary dimension across the entrances of the channels. Consequently, the second dimension can be considered as a couple of parallel positioned separation capillaries. An important advantage of this approach is that it is completely comprehensive in contrast to the previously reported system. Figure 9 presents a schematic diagram of this 2D-CZE/CGE system. Figure 10 shows the optimization results of a series of separations of a sample containing model peptides using different channel fillings: buffer and a series of gel-fillings with concentration varying from 5%T to 50%T (the total concentration of monomer in the gel) at a constant degree of cross-linkage of 3.3%. The sample mixture consists of a number of peptides: Leu-enkephalin, Met-enkephalin, angiotensin II and some smaller peptides consisting of two or three amino acids. These peptides are not separated in the CZE dimension. In free solution channel electrophoresis, this peptide mixture is separated into two poorly shaped bands. No improvement was found for a variety of buffer solutions in different pH ranges. For the peptides, only the channels with a high concentration of gel show significant separation efficiencies especially for the small peptides.

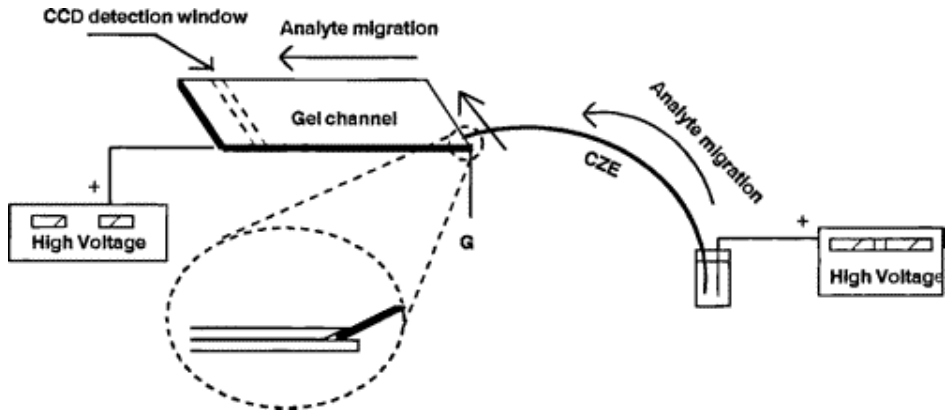

Figure 9. Schematic diagram of the CZE-CGE separation system showing the CZE capillary, gel channel, interface and the power supply arrangement [70]. G is the ground.

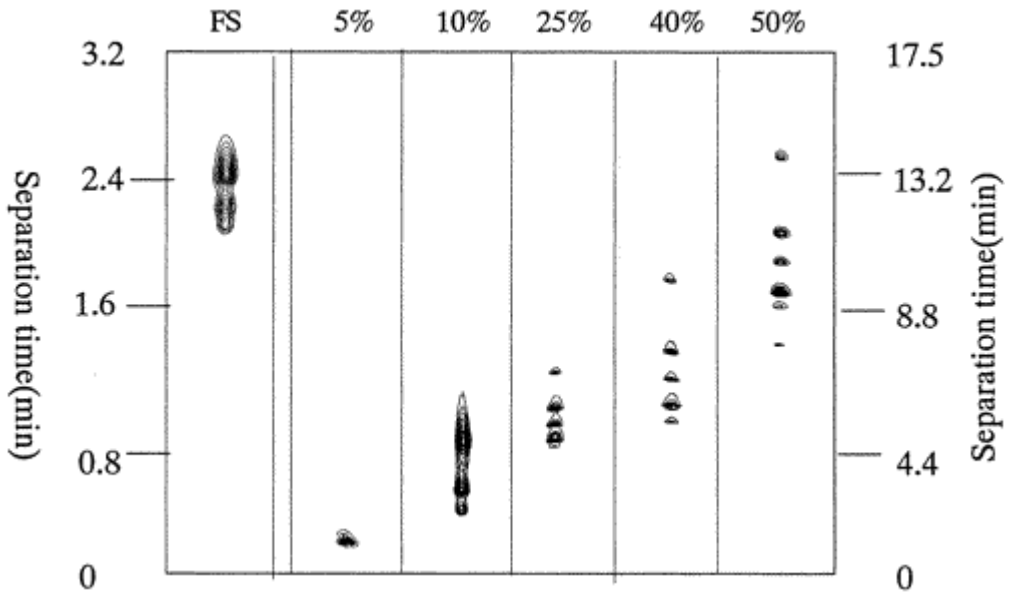

Figure 10. Comparison of free solution (FS) and gel-filled channels for the separation of a series of five labeled peptides of between 2 and 8 residues. The total concentration of monomer in the gel (%T) is shown in each electropherogram. The concentration of cross-linking agent (%C) is 3.3% in all cases [70].

4.2.4. Coupling of MEKC with CZE

The coupling of MEKC with CZE is favorable because of its orthogonality [72]. It resembles the orthogonality of a RPLC-CZE system. Michels et al. developed a system consisting of submicellar capillary electrophoresis coupled to CZE for the detection of proteins, labeled with a fluorogenic reagent. The transverse flow-gated principle of Jorgenson and co-workers [44] was used. The developed system differs from that of Jorgenson in three important respects. First, a CE in both dimensions was used, which eliminates the use of chromatography, with its associated pumps and large sample volumes. Second, the entire fraction is transferred from the first dimension to the second, eliminating the loss of sample in the flow-gated interface. Third, an extremely high sensitive fluorescence detection is used, which allowed the detection of zeptomoles of proteins [72], however the amount of sample which can be injected is smaller.

4.2.5. Microfluidic devices

The number of reports concerning microfluidic devices (Chips, micro total analysis system or μTAS) has increased dramatically since the initial concept was introduced some years ago [73, 74]. In particular, microchip CE devices have been shown to offer many advantages over conventional CE systems: parallel channels in one device, reduced amounts of reagents and waste, increased

precision and accuracy and the potential for disposable devices. In addition, lower voltages are required with microchip CE due to the short channels that are employed for the separation. This enables the use of smaller power supplies, leading to portable analysis systems [73]. Recently a couple of reviews were published concerning various aspects of chip technology. One is dealing with sample pretreatment on microchips [75], others review sample introduction [76], cyclic electrophoretic and chromatographic separation methods [74] and theory and technology of μTAS [77]. Two reviews report various applications of chips [78, 79], including peptide and protein analysis. Although this chip technique is promising, there are only three reports, from Ramsey and co-workers, about 2D electrophoretic applications for the determination of peptides or proteins so far [80-82]. A microfabricated fluidic device was developed that combines micellar electrokinetic chromatography and high-speed electrophoresis for the 2D analysis of peptides. Second-dimension analyses were performed every few seconds, with total analysis times of around 15 min for tryptic peptides. The peak capacity of the two-dimensional separations had been estimated to be in the 500-1000 range. The orthogonality of the separation techniques, an important factor for maximizing peak capacity and resolution, was verified by examining each technique independently for peptide separations. The two-dimensional separation strategy was found to greatly increase the resolving power over that obtained for either dimension alone [80]. This system was further improved [81, 82]. The chip described in ref. [82] enables MEKC separations in a 19.6-cm-long serpentine channel. Subsequently, the peptides were rapidly sampled into a 1.3-cm-long second-dimension channel, where they were separated by CZE. The contributions to band broadening in the channels was further minimized. Analysis of rhodamine B injections routinely produced plate numbers of 230 000 and 40 000 in the first (MEKC) and second (CE) dimensions, respectively, corresponding to plate heights of 0.9 and 0.3 μm. The electric field strengths were 200 V/cm for MEKC and a relatively high 2400 V/cm for CE. In analysis times less than 15 min, two-dimensional separation of bovine serum albumin tryptic digest produced a peak capacity of 4200 (110 in the first dimension and 38 in the second dimension). Figure 11 shows the 2D separation of a bovine serum albumin tryptic digest. The theoretical projections of the 2D data onto each axis are also shown. The system was used to identify a peptide from a tryptic digest of ovalbumin using standard addition and to distinguish between tryptic digests of human and bovine hemoglobin [82].

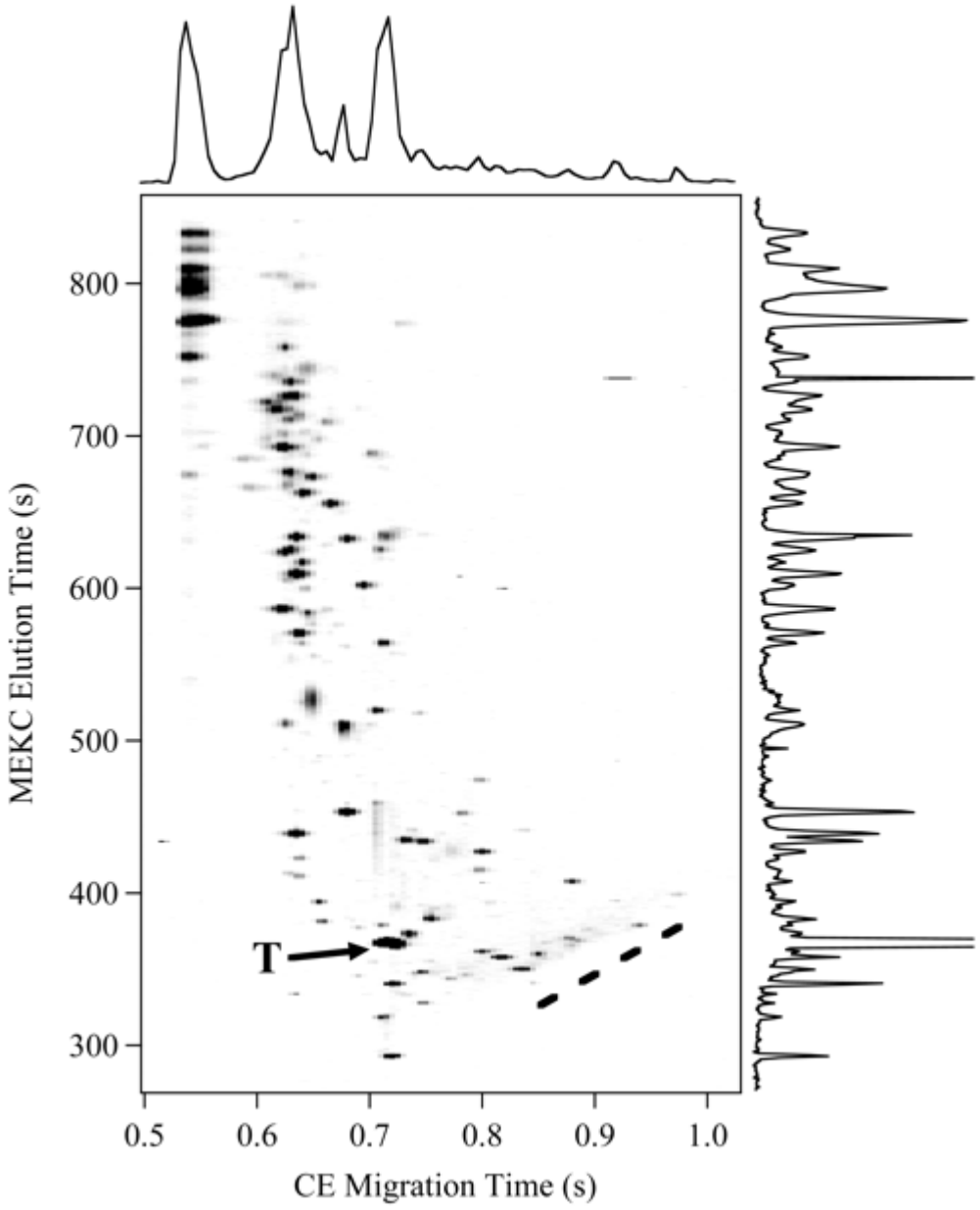

Figure 11. 2D separation of a bovine serum albumin tryptic digest. The projections of the 2D data onto each axis are shown. Spots marked with a "T" represent unreacted 5-TAMRA dye. The dotted line illustrates correlation in the separation mechanisms [82].

From Table III, a number of conclusions can be drawn. So far, no CE-CE system has been used for quantitation of peptides or proteins. It should be noted that a sample pretreatment procedure consisting of a desalting procedure, is nearly always necessary before using these systems, in contrast to the systems starting with a LC mode. Chip technology is still further improved, resulting in increased separation power in very short time. Using chip technology, the transfer of effluent from the first dimension to the second dimension can be performed with limited band broadening.

5. Conclusions

For protein and peptide mapping a semi-quantitative detection is often used, while comprehensiveness and selectivity of the separation system is very important. For quantitation of a certain peptide or protein it is essential to develop a validated sensitive method, while a heart-cut system can then have sufficient selectivity. Generally, the more or less direct injection of biological samples and the speed of the separation system are important. For the development of an on-line multidimensional system to determine peptides and proteins some general strategies can be given. If samples containing considerable amounts of salts have to be injected, SEC or RPLC separation in the first dimension is favorable, while either chromatographic or electrophoretic separation in the second dimension can be used, depending on the nature of the peptides or proteins (lipophilicity, size, charge, etc.). If direct injection of biological samples is required, SEC is a very good choice as the first separation dimension. Combinations with RPLC, IEC or electrophoretic techniques result in systems with large selectivity.

For quantitation purposes LC-LC, LC-CE and CE-CE combinations can be used, with on-line coupling of suitable detection systems (LIF, MS). If target analysis of low–concentrated peptides or proteins is the aim, a (pre)concentration step has to be included in the system. Affinity chromatography seems first choice when low concentrations of specific peptides or proteins must be determined. However, for such a system antibodies should be available for immobilization Trapping on a micro column or concentration on the top of a chromatographic column in the second dimension and stacking in the electrophoretis (for charged compounds is often used.

For high-throughput analysis, for instance in proteomics, it is necessary to separate the compounds at a high speed and combination with tandem MS is mandatory if the system must be employed for profiling purposes. Although, electrophoretic separation techniques in many cases give fast separations, (nano-)LC-CE (FCE) coupling should be further developed. In such a system both dimensions enable separations within minutes and seconds, respectively, enabling the analysis of large numbers of samples within a short period of time. Coupling of CE with MS including different ionization techniques is now becoming routine. For a universal application of fluorescence detection derivatization of the analytes may be necessary, which is a limitation.

Relatively new approaches, such as CE-CE, may have the potential to supersede the conventional LC-LC techniques in some cases, However the LC-LC systems are still much easier to develop, in particular if only a heart-cut system is required. The on-line addition of a MS detector to a LC-LC system can be achieved more easily as well, compared to a LC-CE or a CE-CE system. However, a LC-CE system often seems to be more orthogonal and selective than LC-LC, but generally the

sensitivity is less because only a part of a LC fraction can be introduced into the CE. If a comprehensive system is required, a LC-CE system may also have the advantage of a rapid CE step but such a system is still less robust. In CE-CE, which can have a large orthogonality as well, direct injection of biological samples is generally not possible unless a simple desalting procedure is applied. Using LC-LC or LC-CE direct sample injection is sometimes possible. By use of chip-technology very fast separations can be achieved. Chip-technology is now becoming available with on-line MS detection. If chips will be developed in such a way that direct injection of biological sample is possible with the addition of an on-line desalting step, e.g. with a hollow fiber, a very powerful system will be obtained.

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Chapter 2

On-line sample preconcentration in capillary electrophoresis, focused on the determination of proteins and peptides: a review

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Abstract

Interests in new peptide and protein drugs with therapeutical activity in fairly low concentrations are increasing rapidly. Compared to most of the conventional drugs, for determination in biological matrices the peptide and protein class demands sensitive bioanalytical methods. Capillary zone electrophoresis has high separation efficiency, however and especially when trace-level concentrations have to be determined, the analyte detectability in units of concentration is often insufficient. A desired increase in concentration sensitivity using CZE can be found by introducing analyte enrichment procedures prior to separation. This overview highlights the possibilities of on- or in-line preconcentration procedures in combination with a CZE-separation, focused on the determination of peptides and proteins. The discussed methods, including sample stacking, field amplified injection, isotachophoresis, solid phase extraction, membrane preconcentration, electro-extraction, supported liquid membranes, hollow fibers, immuno-affinity and molecularly imprinted polymers technology preconcentration are categorized in electrophoresis-based and chromatography-based preconcentration. The chromatography-based preconcentration is subdivided in low specificity and high specificity methods. A number of preconcentration methods are available, however this paper demonstrates that various compounds in different media (aqueous solutions, urine, and plasma) require different preconcentration systems. The preconcentration techniques of first choice in general seem to be solid phase extraction and membrane preconcentration, because of their high concentration ability, multi-applicability, relative simplicity and clean-up capability. For the future, hollow fibers seem to hold a great potential as preconcentration technique, yielding high concentration factors, using simple designs.

New techniques, such as hollow fibers, molecularly imprinted polymers technology and supported liquid membranes may have the potential to supersede the conventional preconcentration techniques in some cases. The larger the arsenal of preconcentration-

techniques becomes, the more efficient peptides and proteins may be analyzed in the future. These techniques, in some cases, require pre-clean up procedures, to ensure the purity of the samples to concentrate.

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1. Introduction

Today, the availability on the market of new peptide and protein drugs with therapeutical activity in fairly low concentrations increases rapidly. Compared to most of the conventional drugs, for determination in biological matrices, the peptide and protein class demands sensitive bioanalytical methods. As structural differences between endogenous peptides and proteins sometimes are very small, the bioanalytical methods have to be selective as well. Another reason for the increasing interest in the development of selective and sensitive methods is the challenge for many researchers to establish the peptide profiles of healthy biosystems and to study modifications of these profiles in case of diseases [1]. Most of the present analytical methods for quantitation of peptides and proteins in biological matrices consist of a reversed-phase liquid chromatographic (RP-LC) or a capillary zone electrophoretic (CZE) separation method. However, the large size and the high hydrophilicity of protein drugs complicate the separation of these drugs from endogenous peptides and proteins using RP-LC. Another disadvantage of RP-LC is the frequent use of denaturing modifiers, contrarily to CZE. The main advantage of CZE over RP-LC is the much higher separation efficiency, however, especially when trace-level concentrations have to be determined, the analyte detectability in units of concentration is often insufficient. A number of sensitive detection techniques other than UV-detection have been coupled to CZE to enhance the sensitivity. These methods include laser induced fluorescence (LIF) and various mass-spectrometric (MS) detections [2]. The integration of these techniques with CZE frequently needs appropriate interfaces, whereas also this type of detection is rather expensive. Therefore, the possibilities to overcome restrictions on the detector side are limited. Consequently, the desired increase in concentration sensitivity has to be found by introducing analyte enrichment prior to separation. The sensitivity limit using UV-Vis detection is typically found to be approximately 10^{-6} M . With adequate sample concentration (typically > 10 μg/mL for peptides), an injection volume of 1-5 nL (the usual injection volume for a CZE-system) is sufficient for detection. The preconcentration can be performed manually (off-line), via connecting tubing that transport directly a stream of liquid to the CZE (on-line) or fully integrated in the CZE (in-line). The on- and in-line combinations of preconcentration with CZE separation for the quantitation of peptides and proteins in a complex matrix enable a significantly faster analysis in comparison with an off-line combination.

In the next paragraphs an overview will be given of the possibilities of on- or in-line preconcentration procedures in combination with a CZE-separation, focused on the determination of peptides and proteins. The discussed methods will be categorized in electrophoresis-based and chromatography-based preconcentration. The chromatography-based preconcentration is subdivided in low specificity and high specificity methodology.

2. Electrophoresis-based preconcentration

There are three main electrophoretic preconcentration mechanisms: sample stacking, field amplified injection and isotachophoresis.

Sample stacking is based on the principle that the velocity of an ion in CZE is the product of its mobility and the electric field strength. If the conductivity of the sample plug is lower than that of the CZE buffer, the electric field strength in the sample plug is higher, compared to the CZE buffer. Consequently, the velocity of the ions in the sample plug is higher than in the CZE buffer and the analyte will be focused in the running buffer if the analyte solution is sandwiched between the running buffer while CZE is performed. Analyte stacking will result in an increased signal output and an improved concentration limit of detection (CLOD) [2 - 7]. For a maximum increase in signal output of about 20-fold, the sample plug has to contain organic solvent to obtain a low conductance. This gives rise to the problem, especially with proteins, that precipitation may occur. Stacking is not suitable as a clean-up technique for biological samples, due to the presence of salts and proteins, but can be used successfully to improve the CLOD for the analysis of peptides and proteins [3, 5, 6, 8] when the low injection volume is a limitation.

The principle of *field amplified injection* (FAI) is similar to that of sample stacking and is based on the difference between the velocity of the analyte in the sample plug and the velocity in the running buffer. The only differences are the injection procedure and the focusing process. This process in FAI is performed when the injection part of the capillary is still in the sample vial and with an applied voltage during injection, while the focusing process in stacking is performed after replacing the sample vial for a buffer vial and during the beginning of the CZE-run. Consequently, the whole sample can be processed using FAI, instead of a limited volume of 70% of the capillary when using sample stacking. FAI procedures can be used for the determination of positively and negatively charged

compounds. The main advantage of FAI is a large preconcentration factor of ca. 100, because the whole sample is processed [2]. The main disadvantages of FAI are the strong influence of the matrix on the conductivity and the discriminating injection. Consequently, FAI is seldom used for the analysis of compounds in biological samples. However, it is used for the analysis of amino acids [9, 10] and organic compounds in aqueous solutions [11], which makes it conceivable that FAI may be applicable to peptides and proteins, although no reports have been published so far.

In *isotachophoresis* (ITP), the analyte containing plug is positioned between two different buffers, the leading electrolyte (LE), containing ions with a higher mobility than the analytes, at the detector side and the terminating buffer (TE), containing ions with a lower mobility than the analytes, at the injection side. ITP is performed mainly in two modes. The first mode is performed using two on-line coupled capillaries. The first one is used for the ITP procedure and the second for the CZE procedure. This mode is called the coupled-capillary ITP (cITP). In the second mode, the same capillary is employed for both the ITP and the CZE procedure, thus enabling an in-line approach. In practice, cITP is not often used because it can be applied to aqueous samples only [2] and needs a complex set up. Therefore, this section is focused on the transient ITP (tITP), the in-line method. The procedure of tITP consists of two steps. In the first step, the analyte is dissolved in the LE, often an ammonium solution, and introduced in the CZE-capillary using hydrodynamic pressure. This step enables the filling of about 70% of the capillary with sample [12]. Subsequently, the inlet of the CZE capillary is placed in a vial containing the TE, normally containing acetic acid. Thereafter, a voltage of ca. 30 kV is performed over the capillary that forces the LE, the analyte and the TE to migrate towards the cathode at the detection side of the capillary. Because of the differences in mobility, the analyte will be focused and concentrated between the LE and the TE. A hydrodynamic backpressure at the detection side of the capillary is necessary to avoid migration of the analyte out of the capillary. In the second step, a normal CZE is performed, using the LE as background electrolyte (BGE). The technique is suitable for the analysis of both anionic and cationic peptides, although not in the same run. Anionic peptides can be determined using either a permanently coated silica CZE-capillary, or a dynamic coating procedure, in which the coating material is dissolved in the BGE [12]. The tITP procedure enables a sample concentration factor of ca. three orders of magnitude in comparison with normal CZE. tITP has been utilized for the determination of both peptides [12-14] and proteins [15]. Today, with tITP a concentration sensitivity about the same as in conventional HPLC can be reached. Consequently, tITP shows great promise of becoming an analytical tool for peptide or protein

analysis. However, biological samples do still need a clean-up prior to the tITP-procedure, because endogenous proteins and especially salts interfere. In Table I an overview is given of the application of preconcentration methods coupled to CZE.

Table I. Preconcentration methods

Method	Usable for clean-up	Concentration ability	Employed to	References
Electrophoresis-based				
Sample stacking	no	<20	amino acids, model proteins/peptides , insulin (general papers)	[46] [8] [3] [5], [6]
FAI	no	100-500	amino acids organic compounds (general paper)	[9], [10] [11] [1]
tITP	no	2-3	adenosine recombinant cytokine fragments amino acids, standard proteins rhIL-3, rhIL-6, basic proteins angiotensin II, gonadorelin tryptic digest cytochrome c, substance P, calcitonine gene-related peptide	[47] [48] [8], [49] [15] [12], [13] [13]
Chromatography-based				
SPE	yes	3-4	model peptides/proteins metallothionein proteolytic proteins angiotensin II, gonadorelin humanized porcine insulin, horse heart cytochroom c MHC I* (general papers)	[50] [51] [52] [17], [18] [18] [19], [23] [54], [20], [16], [2], [53], [15]
mPC	yes	3-4	peptides , MHC I*, β-2 micro globulin, apolipoprotein a1, serum albumin (general papers)	[47], [20] [22] [53], [55]
EE	yes	1000	β-antagonists (general paper)	[39] [1]
SLM	yes		Bambuterol	[25],[26]

Table I continued

Method	Usable for clean-up	Concentration ability	Employed to	References
Hollow fibers	no	1000	cytochome c, lysozyme, ribonuclease A, α-chymotrypsinogen A	[27], [28]
Immuno-affinity	yes	100-500	MHC I* (general papers)	[56] [31], [32], [33], [57], [58]
MIP	yes	unknown	(general papers)	[34], [35], [36], [37]

*) MHC, major histocompatibility complex

3. Chromatography-based preconcentration

Several methods are available for on- or in-line preconcentration, based on different chromatographic mechanisms. These chromatographic mechanisms can be of low specificity or of high specificity. Basically, three methods are available using a low specific chromatographic mechanism: (1) solid adsorptive phase chromatography, including solid phase extraction (SPE) and membrane preconcentration (mPC) [2, 16-24], (2) liquid distribution phase chromatography, including electro-extraction (EE) [2] and supported liquid membranes (SLM) [25, 26] and (3) hollow fibers [27, 28]. Low-specific chromatography is probably the most widely used application of chromatographic retention. Two methods perform a preconcentration based on a high specific chromatographic mechanism: immuno-affinity chromatography [29-33] and molecularly imprinted polymer (MIP) technology [34-37]. Chromatography-based preconcentration prior to CZE has the advantage that the chromatographic mechanism is orthogonal to the electrophoretic mechanism. Consequently, this preconcentration technique can be performed with more selectivity in contrast with electrophoretic-based preconcentrations. With chromatographic techniques the analytes to be concentrated should preferably be neutral and the pH of the system should be selected accordingly. However, in CZE the analytes have to be charged if CZE is going to be used. If a chromatography-based preconcentration method is applied to non-charged analytes, the CZE method can be modified by applying, as an alternative, a micellar system [2].

The following section will discuss the chromatography-based preconcentration more thoroughly, including the applicability to protein and peptide analysis.

3.1 Low-specific chromatographic preconcentration

3.1.1 Solid adsorptive phase chromatography

Solid adsorptive phase chromatography based preconcentration, including on-line SPE and in-line mPC, has mainly one advantage over the formerly described electrophoretic methods: the ability not only to enrich, but also to clean-up the sample. This is extremely useful in the analysis of biological samples such as blood or urine. With SPE and mPC the sample can be purified from interfering and clogging components, such as proteins, and high salt contents, which can disturb the electrophoretic process.

The hardware-setup of a *spPC-CZE-system* consists of a (miniaturized) preconcentration-column or a similar device, containing reversed-phase HPLC-packing material coated with a hydrophobic adsorptive phase, such as C_2, C_8 or C_{18} material. The packing material is kept in place by using frits at each side of the packing. The preconcentration column is directly connected to the CZE-capillary. After loading the solid phase, with sample the system is washed out. Usually, this is performed with normal buffer used in the CZE separation process. In the loading step of the process it is possible to inject much higher sample volumes, contrarily to plain CZE. After washing the sample the eluting step is carried out by injecting into the system a small plug, usually 50-100 nL, of a solvent containig an organic modifier [29]. Subsequently, the CZE separation process is carried out. The use of a modifier-containing plug causes sample stacking in the CZE process, as described previously. Besides some advantages of this method, it is not without limitations. The method will significally enhance the CLOD. However, in practice, the CZE performance is often compromised [38]. The method often results in reduced analyte resolution, peak broadening and substantial component tailing. Analysis time is also longer than with normal CZE. Various attempts have been made to overcome these limitations. One of them is to reduce the size of the solid phase. However, this can cause problems with reproducible reconstruction of small bed volumes of solid phase material.

mPC was developed to remove or, at least decrease, the limitations of SPE. Using a suitable coated or impregnated membrane, usually with C_8 or C_{18} material, it is possible to minimize the bed volume of adsorptive phase at the inlet of the preconcentration capillary. This method proves to be much more efficient than SPE. The EOF is more reproducible, the high adsorptive capacity of the membrane permits injection of volumes higher than with SPE, elution volumes are much lower and this modification leads to a more efficient removal of analytes from the sample. In terms of clean-up the membrane is much less affected by contaminants originating from urine or plasma than the material used in SPE [38]. The membrane is installed in a cartridge that is usually prepared from Teflon tubing. Fused silica and metal (stainless steel or titanium) has also been used to prepare mPC cartridges. The loading, removal and separation processes of mPC-CZE are more or less the same as in spPC-CZE.

With both spPC and mPC, instead of nanoliters, volumes up to 25-100 μL are possible to analyze, with an improvement in detection of about 3-4 orders of magnitude. In a study, using three different types of reversed-phase preconcentration, a limit of detection of 20 pg, using UV detection, could be obtained, corresponding to a 20 μL injection of a 1 ng/mL solution of a model peptide [17]. mPC and spPC are valuable methods of CZE preconcentration, especially for analysis of biological samples. Various publications concerning these methods are listed in Table I.

3.1.2 Liquid distribution phase chromatography

Liquid distribution phase chromatography consists of two techniques: electro-extraction (EE) and supported liquid membranes (SLM) technology.

Electro-extraction (EE) is a miniaturized extraction technique based on both liquid-liquid distribution chromatography and electrophoresis. The difference in polarity of the sample solution and the LE, used in CZE, is used to concentrate the analyte. The analyte has to be extracted from a non-polar phase (e.g. ethyl acetate) to the aqueous running buffer. The extraction normally is followed by an ITP focusing step. The process consists of three steps. In the first step, a capillary filled with leading buffer and a small plug of terminating buffer is placed in the injection vial containing the analyte dissolved in ethyl acetate. A voltage is applied, together with a hydrodynamic counterflow, equal to the electroosmotic flow (EOF),

to avoid injection of a large volume of ethyl acetate into the CZE capillary. The voltage will cause the charged analytes to migrate into the CZE capillary. Next, the capillary is placed in a vial with the terminating buffer and the focusing step is started. After steady-state the capillary is placed in the leading buffer vial and CZE-analysis is started. High concentration factors can be established [39]. However, this method still has to be adapted to the analysis of peptides and proteins. One of the main problems to overcome is the problem of denaturation and precipitation of the proteins in the non-polar phase.

The use of *supported liquid membranes (SLM)* is a relatively new technique. The hardware setup of this method enables a double extraction and concentration of the analyte [2, 25, 26]. The system (Fig.1.) consists of a polypropylene fiber impregnated with a hydrophobic organic solvent (e.g. 6-undecanone). This impregnated polypropylene fiber prevents the mixing of two aqueous extraction phases, a donor phase and an acceptor phase. Both the polypropylene fiber, the donor phase and the acceptor phase are placed in a polymer block.

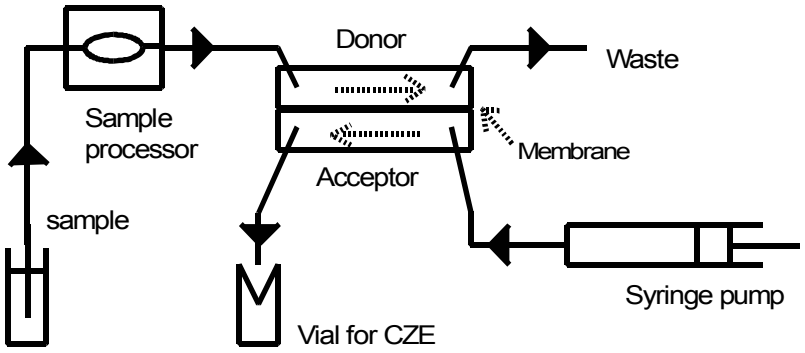

Figure 1. Set-up for supported liquid membranes.

By adjusting the pH of the two phases in such a way that the analyte is uncharged in the donor phase and becomes charged in the acceptor phase it is possible to selectively enrich the acceptor phase of the system with analyte. If basic analytes have to be concentrated, the donor compartment is adjusted to a rather high pH (10-12) and filled with the sample, while the acceptor phase is adjusted to a rather low pH (2-4). Consequently, the basic analytes can easily pass the membrane in their neutral form. Subsequently, they will become charged in the acceptor phase and diffusion back into the membrane will be prevented. During

enrichment the sample is pumped through the donor phase compartment. Subsequently, the plug of acceptor phase, with a volume of about 50 μL, is further concentrated electrophoretically using a stacking procedure, whereafter normal CZE is carried out. The procedure for acidic compounds is similar. However, a low pH has to be used in the donor phase and a high pH has to be used in the acceptor phase.

So far, only one report of this technique appeared in the literature: the determination of the basic drug bambuterol in plasma with UV detection [24]. The CLOD was 2 nM, with a linear response over almost two-orders of magnitude. The impregnated polypropylene fiber can be used for 6 hours without becoming exhausted. This technique has some potential, because the system can result in a selective clean-up. The pH of the donor and acceptor phases can be accurately set and different liquid phases can be used to selectively clean-up the biological samples. However, it is not clear if this technique can be made applicable for peptide and protein analysis. The long analysis time is a disadvantage. Also, the manual handling of the procedure is still a problem if this technique has to be made suitable for routine analysis. Various applications of liquid distribution phase chromatography are presented in Table I.

3.1.3 Hollow fibers

Hollow fibers have already been used in different applications, for instance for cross-flow ultradialysis [28]. Recently, investigation has been done whether these hollow fibers can also be used to concentrate a dilute sample [27, 28]. The technique is based on the transport of water out of the fiber through the pores on its wall. By choosing a suitable cut-off molecular weight value of the hollow fiber, also some purification can be achieved.

An experiment proved the suitability of a hollow fiber as a preconcentrator for protein analysis [27]. A short hollow fiber with a suitable molecular weight cut-off value is connected to the inlet end of a CZE capillary (Fig.2). An injection electric field is applied across the hollow fiber. Proteins electromigrate into the hollow fiber from a sample vial, according to their charge. Since the proteins cannot pass the hollow fiber pores, they are concentrated at the inlet of the capillary. After a concentration period, the concentrating electric field is turned off. Subsequently, the separating electric field is switched on and normal CZE is carried out. The study showed, with a 60 second concentration time, a 1000-fold increase in signal with UV-detection in the analysis of some model proteins.

This method is a promising one in the trace analysis of proteins. Fast and efficient on-line preconcentration is achieved with removal of small molecules, including salts, which can disturb the CZE-separation. Proteins with different sizes (by choosing a suitable molecular-

weight cut-off value of the hollow fiber), both cationic and anionic (by reversing the electric field), can be concentrated. However, biological samples may need some pre-handling to prevent too much stacking of endogenous proteins at the inlet of the capillary. Table I includes applications of hollow fibers in protein analysis.

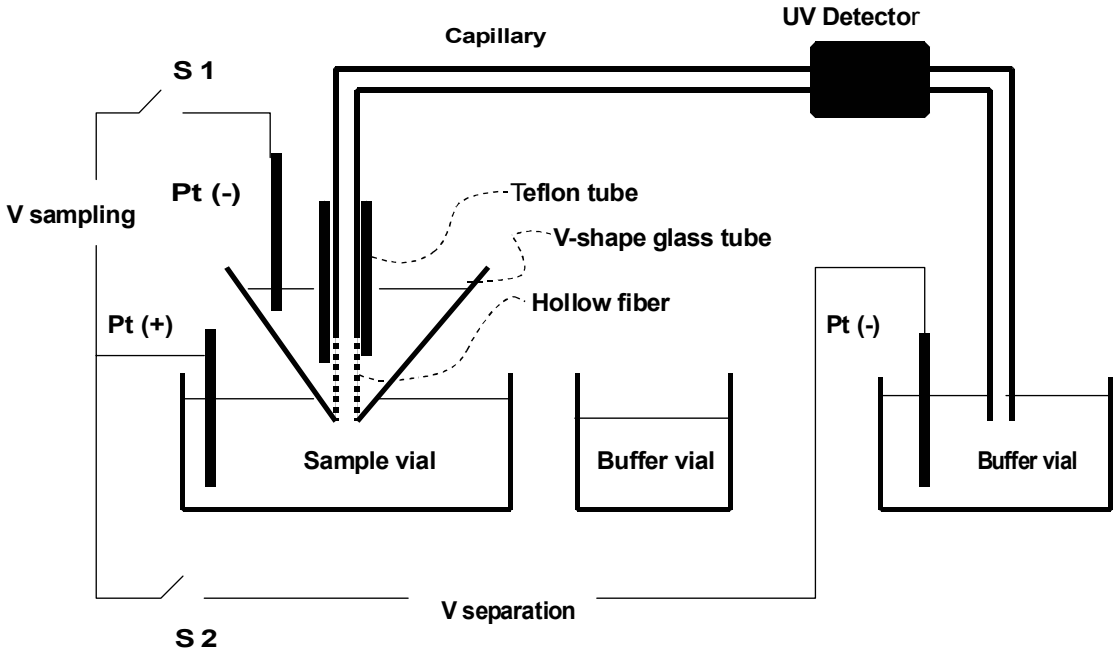

Figure 2. Experimental system for the on-line electrophoretic concentration of CZE with a hollow fiber.

3.2 High-specific chromatographic preconcentration

Although many low-specific chromatography-based preconcentration metods have been proven to be successful, high-specificity chromatography can sometimes be more appropriate, if very selective isolations have to be made.

3.2.1 Immuno-affinity chromatography

The standard setup of an immuno-affinity preconcentration system contains a solid support, immobilizing the antibody. The enrichment chamber is connected on-line with a CZE-system. The enrichment is achieved by pressurizing the sample through the preconcentration system, in which the analytes will bind to antibodies, or receptors. In a subsequent step, the

analytes are released from the receptors by a small volume of eluent. The eluting solution, who's goal primarily is to achieve dissociation of the analyte-antibody-complex, can be composed of a small plug of an organic solvent, a buffer with a pH where the binding is reduced, a solution with increased ionic strength, or a buffer containing special additives, depending on the compound to be analyzed [30]. In this approach, a larger injection volume, up to 50 µl, can be introduced into the CZE-system, thus effectively lowering the CLOD.

The different types of preconcentrators differ in the solid supports retaining the antibodies and the type of antibody used. The main goal of developing a solid support always seems to be the creation of a maximum surface to immobilize the antibodies with a minimum of hydrostatic resistance. Consequently, the capacity of the preconcentration chamber has to be high, where at the same time the chance of clogging and peak broadening has to be low. Several types of solid supports are currently available.

The simplest enrichment chamber is made of a portion of the CZE capillary containing the antibody on its wall. A second portion of the capillary is used for normal CZE. An advantage of this method is the lack of frits and, consequently, the decreasing chance of clogging and peak broadening. The main disadvantage is the limited surface area, resulting in limited enrichment capacity [30].

A second preconcentration system uses a membrane, impregnated with a suitable receptor/antibody and placed in a Teflon cartridge. This technique is also used in other preconcentration systems. This mode of preconcentration is mostly used for enrichment, clean-up and desalting of biological fluids in low concentrations. A limitation of the method is again the restricted surface area. The concentration capability of this design can be from 100- up to 500-fold [30].

A third on-line preconcentration system uses HPLC-packing beads, retained by a single frit at the end of the enrichment chamber. An increase of 200-fold in detectability can be achieved. Problems are the use of frits, resulting in a less uniform flow, and the packing material that may cause blocking and clogging of the capillary. Instead of HPLC-packing material, solid, porous, irregularly shaped beads made of glass or plastic, can be used as well. Then a much larger surface area is created, enabling a 200- to 5000-fold increase in signal [30].

Another on-line preconcentrator cartridge design was manufactured without the use of frits or membranes. It consists of a capillary with a small quantity of magnetic beads containing immobilized biomolecules. The plug of beads (2-3 mm) is prevented from moving by a magnet placed in the cartridge of the CZE-system. The beads can be replaced after a run, therefore regeneration of the solid support is not necessary [30]. One of the protocols used for

this cartridge design was employed to quantitate an antigen (mouse monoclonal antibody) using a suitable antibody. Only femtomoles of material were required.

The last currently available preconcentration chamber, also manufactured without the use of frits, beads or membranes, consists of a bundle of small-diameter capillaries. The main role of every microcapillary is to serve as a solid support attachment site for the antibodies. A uniform electroosmotic flow, with a decreasing chance of clogging the chamber, is the main advantage of this method [30].

When immuno-affinity chromatography was first used for preconcentration purposes, the method suffered from low binding capacity and limited specificity due to the quality of the (polyclonal) antibodies [29]. However, the introduction of monoclonal antibodies began a new era, allowing columns with any desired specificity and high binding capacity, requiring only mild elution conditions. Various publications concerning immuno-affinity chromatography-based preconcentration methods are listed in Table I.

3.2.2 Molecularly Imprinted Polymer technology

Molecular imprinted polymers (MIPs) have drawn much attention in the field of chemical analysis. Various researchers studied the applicability of MIPs in protein or peptide analysis [34-37]. In the field of peptide/protein analysis, this method may be useful as preconcentrating step.

Molecular imprinting is a method to create receptor-like binding sites for analytes (templates). The binding sites are made by polymerization of monomers with affinity for functional groups in the template, for example, a drug. The polymerization of these monomers is performed in the presence of the template. Subsequently, the template is removed and special cavities with affinity for the template arise, assuming that the binding between polymer and template is more or less reversible. The MIPs can be used for the selective rebinding of the template. Special attention is drawn to the fact that MIPs are proven to be useful as chiral stationary phases (CSPs) [40-44], enabling their employment for the concentration of enantiomers. Although MIPs are used as stationary phases in HPLC, no publications have been found dealing with MIPs in on-line preconcentrating techniques. However, MIPs have been used for off-line SPE (MIP-SPE) successfully [36], although some problems with remaining template species in the column arise, reducing binding capacity and contaminating the sample. This can be avoided by extensive washing procedures.

The use of MIPs in chromatographic techniques has mainly been focused on the separation of enantiomers. Studies dealing with separation of peptides and proteins are limited. Some techniques have been described, but in the case of proteins, surface imprinting seems to be promising [37]. However, MIP technology is mostly performed in the presence of non-polar organic solvents. Consequently, when using MIP technology for the preconcentration of proteins, preparation of the MIPs has to be possible in water.

Some proteins, including albumin, immunoglobulin G, lysozyme, ribonuclease and streptavidin are employed in a technique using mica as a suitable surface and disaccharides for the polymerization technique [37]. Some investigations dealing with peptide imprinting have also been reported [34, 45]. The use of MIPs in chromatographic techniques supposedly induces the development of preconcentration methods based on MIP technology.

The advantage of MIPs is the possibility of creating robust, non-expensive and high-selective recognition sites for theoretically any analyte. However, it needs still extensive investigation of research to apply this technique in peptide/protein PC-CZE. Table I contains references to papers related to the use of MIPs for preconcentrating purposes.

4. Conclusions

Peptides and proteins are becoming increasingly important in today's pharmacology and, obviously, in analysis. CZE provides a technique to analyze these compounds and PC-CZE may be able to overcome to a great extent the sensitivity limitations of plain CZE.

A number of preconcentration methods are available, however this paper demonstrates that peptides and proteins in different media (aqueous solutions, urine, and plasma) require different preconcentration systems. When a sample contains high amounts of salt for example, only tITP or sample stacking is not sufficient or even possible and techniques like immuno-affinity chromatography or SPE have to be used. However, when the compounds are dissolved in an aqueous solution, more simple methods such as tITP are applicable.

The PC-techniques of first choice seem to be SPE-PC and mPC, because of their high concentration ability, multi-applicability, relative simplicity and clean-up capability. In the future, hollow fibers seem to hold a great potential as PC-technique, yielding high concentration factors and using simple designs.

New techniques, such as hollow fibers, MIPs and supported liquid membranes may have the potential to supersede the conventional PC techniques in some cases. The larger the arsenal of PC techniques becomes, the more efficient peptides and proteins may be analyzed in the

future. In some cases, however, pre-clean up procedures are required to ensure the purity of the samples to concentrate.

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Chapter 3

Development of an on-line size exclusion chromatographic - reversed-phase liquid chromatographic two dimensional system for the quantitative determination of peptides with concentration prior to reversed-phase liquid chromatographic separation

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Abstract

Complex samples, containing endogenous peptides require analytical methods with high sensitivity and selectivity to enable reliable analysis of these compounds in biological samples. In a number of cases the selectivity of mono-dimensional separation systems is not sufficient. In order to improve the separation efficiency two- or multi-dimensional systems can be the solution.

The present study aims the development and validation of an on-line two-dimensional separation system, using size exclusion chromatography (SEC) and reversed-phase liquid chromatography (RP-LC), to *quantitate* structurally related peptides in the presence of large proteins such as albumin in contrast with the available coupled-column systems which are focused on the *qualitative* determination of peptides. As model peptides a number of enkephalins have been chosen. Albumin was added to mimict a real biological sample.

If the SEC dimension, the RP-LC dimension and the interface have been chosen properly, the entire peak, eluting from the SEC column and containing the enkephalins, can be trapped in a loop after complete separation from albumin. Subsequent separation of the enkephalins is achieved in the RP-LC dimension. Detection is performed using UV absorbance.

The procedure is validated with respect to *recovery*, *linearity* and *intra-* and *inter-day precision*. The lower limit of quantitation is equal for all enkephalins studied and determined to be 5 ng at a signal-to-noise ratio of 5. Based on the injection volume of 5 μL in the first dimension a concentration limit of quantitation of 1000 ng/mL has been established.

Although the developed on-line coupled two-dimensional separation system is suited for the quantitation of various enkephalins in the presence of albumin with a satisfactory linearity, precision and recovery, the method lacks still the sensitivity to allow measurements of endogenous enkephalins in a biological matrix. More sensitive detection methods or miniaturization of the system are being tested currently. The value of the present study, however, is that it demonstrates the potency of the

two-dimensional system for quantitative purposes in peptide profiling in order to establish the normal peptide profiles of healthy biosystems and to study quantitative modifications of these profiles in case of diseases.

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1. Introduction

Today, peptide profiling of biological samples is a challenge for many researchers in order to establish the common peptide profiles of healthy biosystems and to study (quantitative) modifications of these profiles in case of diseases. Generally, peptides are assumed to be active in fairly low concentrations. The bioanalysis of these peptides has become rather important. Selective analytical approaches involve the separation of the peptides under investigation from interfering compounds with structural similarity. As structural differences between peptides and proteins sometimes are very small, these separations may be tedious. Complex samples, containing endogenous peptides require analytical methods with high sensitivity and selectivity to provide a reliable determination of these compounds. Most of the analytical methods present for quantitation of peptides in biological matrices consist of a solid-phase sample pretreatment, followed by an off-line instrumental separation [1-3]. 2-D gel electrophoresis is also widely used for peptide and protein profiling [4-7]. However, this technique is tedious to use in routine analysis.

The combination of two different separation principles has proven to be a powerful tool for the determination of peptide/protein mixtures [8-11]. Sometimes, analyses are carried out in more than one analytical dimension in order to achieve the required resolution and/or selectivity [8-12]. An increase in resolution and selectivity can be obtained only, when the analytical dimensions are based on different separation mechanisms [13]. In addition of being orthogonal to preceding dimensions, a subsequent dimension should not reverse the resolution achieved by the previous ones [11, 13]. Coupled systems require an appropriate interface between the systems. In a coupled technique a loop can be used to trap the fraction of interest, where after a switching valve is employed to reinject this fraction. The on-line coupling of two separation systems for the quantitation of peptides in a complex sample enables a significantly faster analysis in comparison with an off-line solid-phase extraction. This because in off-line techniques transportation of the analytes of from the first to the next dimension has to be performed manually [12]. Multi-dimensional on-line coupled analytical techniques can therefore be advantageous in the assay of smaller peptides in complex biological matrices such as plasma and tissues. If the separation of the complete sample in both dimensions is achieved, the on-line multidimensional system used for peptide profiling is considered to be completely multidimensional.

Another possibility is the use of a heart-cut system. A heart-cut system separates only a fraction, retrieved from the first dimension, in the second dimension. If the time required for making a chromatogram on the second dimension will be the same as for filling a loop with effluent from the first dimension, a complete two-dimensional separation method may be possible. This can only be achieved if adjustments in the flow rates and in the dimensions of the columns are made. A system that fulfills these requirements is described by *Bushey* and *Jorgenson* [11]. The employment of coupled columns, including heart-cut and complete two-dimensional separation systems is not new. Column switching systems have been used for the determination of various compounds [14-21]. The heart-cut applications mainly exist of on-line sample pre-treatments, including sample enrichment for the quantitation of drugs, whereas the application as a tool to combine orthogonal separation mechanisms is less common, in contrast to the complete two-dimensional separation systems applied for protein / peptide profiling, which combine mainly orthogonal separation mechanisms, *e.g.* the combination of a size exclusion chromatographic separation and a reversed phase chromatographic separation [22-27]. However, these systems have not been employed for quantitative purposes.

The present study aims the development and validation of an on-line coupled two-dimensional separation system based on the heart-cut principle, using size exclusion chromatography (SEC) and concentration prior to reversed-phase liquid chromatography (RP-LC), to separate and quantitate a group of structurally related peptides in the presence of proteins such as albumin, which are the most interfering compounds in biological samples. Although a heart-cut system is not novel, the application in *peptide profiling* and *peptide quantitation* is. As model peptides a group of enkephalins have been chosen. Fig. 1 shows their structures and illustrates their degree of similarity. The SEC dimension, the RP-LC dimension and the interface must be chosen properly to trap the entire peak containing the enkephalins, that elutes from the SEC column in a loop after complete separation from albumin. This is possible since the enkephalins have approximately the same size. Subsequently, concentration and separation of the enkephalins must be achieved in the RP-LC dimension.

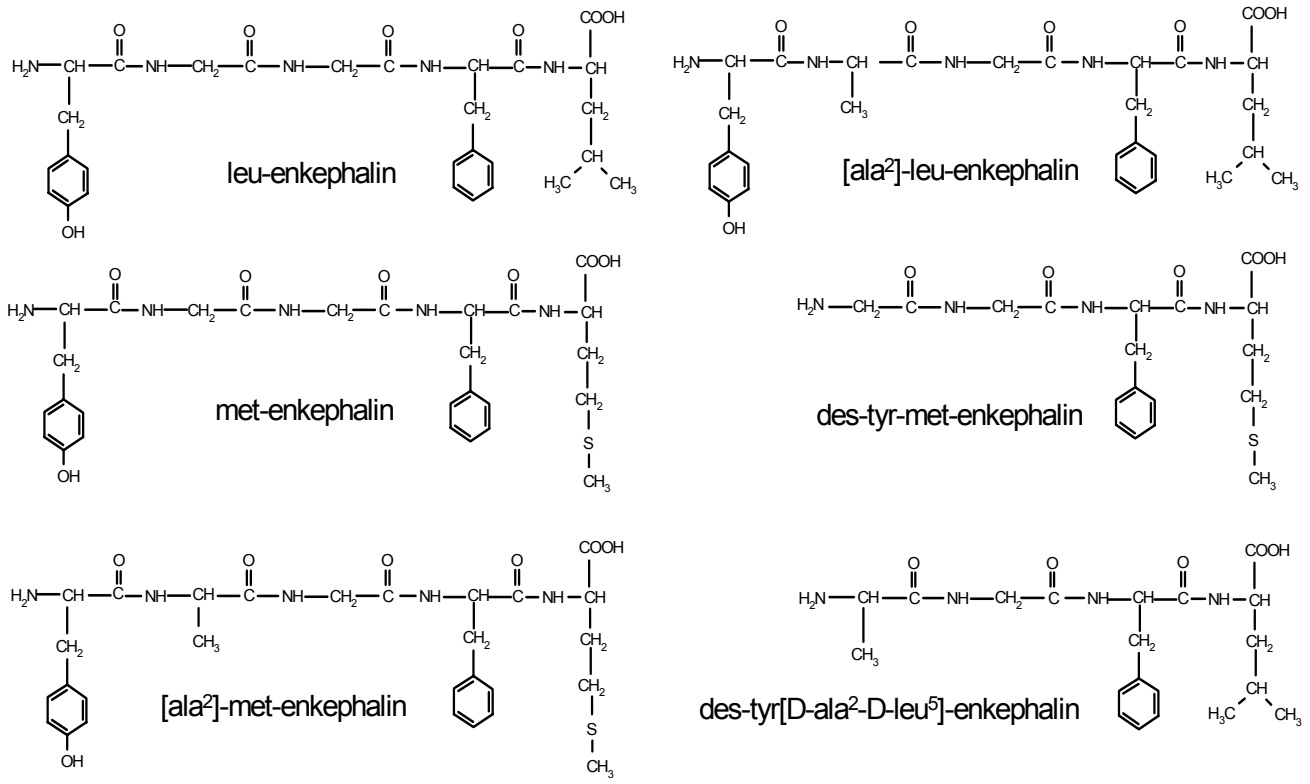

Fig. 1. Structures of the employed model enkephalins

The validation is especially focused on the quantitative aspects of the system, whereas, in general, the existing systems for peptide / protein profiling mainly focus on detection of the compounds only. In contrast with these methods, the heart-cut system, presented in this study, will only trap the fraction from the first column, containing the enkephalins , and reinject this onto the second column, thus facilitating a quantitative approach.

2. Experimental section

Chemicals

Glacial acetic acid, ammonium acetate and acetonitrile (analytical grade) were purchased from Merck (Darmstadt, Germany). The enkephalin peptides (leu-enkephalin, met-enkephalin, des-tyr[D-ala^2-D-leu^5]-enkephalin, des-tyr-met-enkephalin, [ala^2]-leu-enkephalin, [ala^2]-met-enkephalin) came from Sigma (St Louis, MO, USA) as well as bovine albumin, purified by cold alcohol precipitation and essentially globulin free.

Instrumentation, chromatographic conditions

SEC-system

The first dimension (SEC) consists of a 4.6 mm i.d. × 30 mm stainless steel Phenomenex column packed with hydrophilic bonded silica (Biosep SEC-2000©). The particle size is 5 µm and the pore size 145 Å (Bester, Amsterdam, The Netherlands). Both the exclusion and the inclusion volumes of the column are 0.2 mL. The column is thermostrated at 40°C in a Shimadzu CTO-10AS column oven ('s-Hertogenbosch, The Netherlands). The mobile phase in this dimension (solvent A) is demineralized water with 0.01% w/w glacial acetic acid and 0.125 % w/w ammonium acetate (pH 5.8) and is degassed before use. The mobile phase is supplied by a Waters Associates Model M6000 pump (Milford, Massachusetts, USA) (Fig. 2, pump 1) at a flow rate of 0.2 mL / min. The detection is performed by a Shimadzu diode array detector Model SPD-M10 ('s-Hertogenbosch, The Netherlands) (Fig. 2, detector 1). The samples (5µL) were injected onto the SEC column with a Shimadzu autosampler, Model Sil-10A ('s-Hertogenbosch, The Netherlands). Initial method development work was made with the SEC column outlet connected to a Separations UV detector Model 757 (H.I. Ambacht, The Netherlands). The wavelength was set at 215 nm.

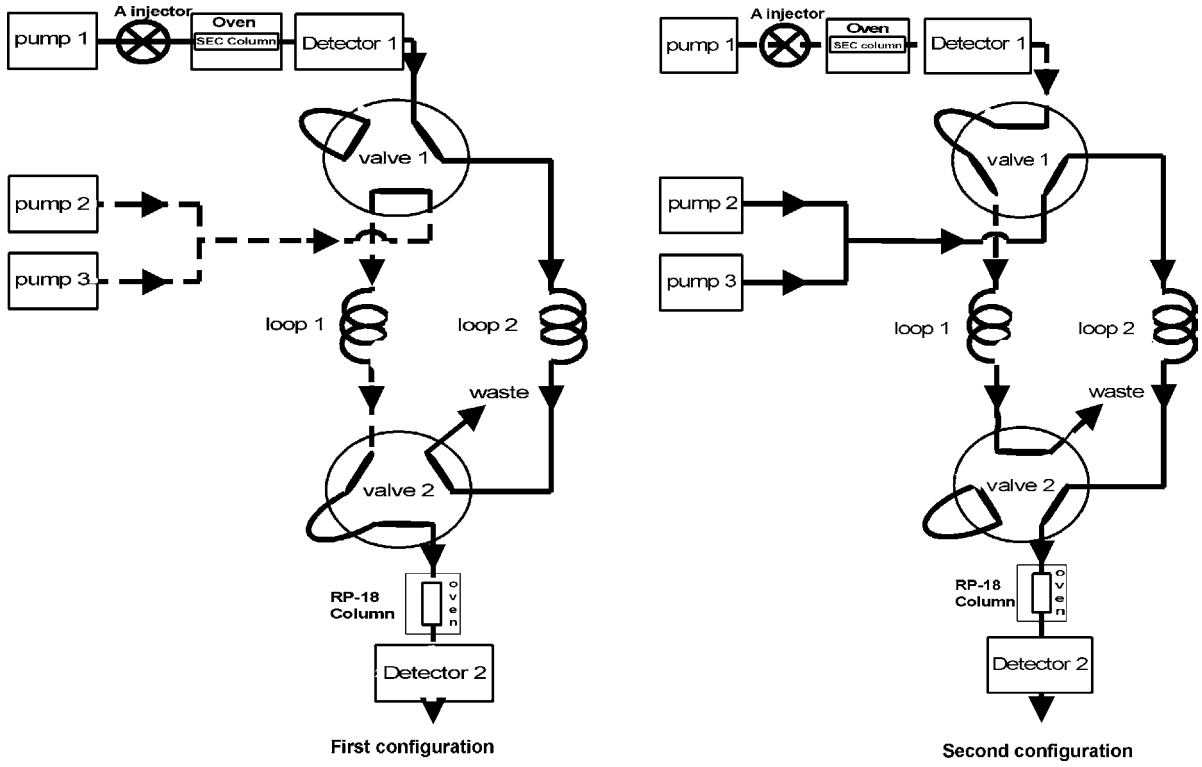

Fig. 2. Instrumental set up of the coupled system in the two positions of the six-port valves.

RP-LC-system

The second dimension (RP-LC) consists of a 2.0 mm i.d. × 125 mm stainless steel Phenomenex column, packed with C-18 bonded silica (Lichrosphere). The particle size is 3 μm and the pore size 100 Å (Bester, Amsterdam, The Netherlands). Chromatography is performed with a binairy system using two solvents. Solvent B is demineralized water with 0.05% w/w glacial acetic acid and 0.0625 % w/w ammonium acetate. The pH of solvent B is 4.8. Solvent C is solvent B to which 60% w/w acetonitrile is added. The solvents are degassed before use. The binairy system is performed using two pumps (Shimadzu SCL-10A pump,'s-Hertogenbosch, The Netherlands) (Fig. 2, pumps 2 and 3). The separation is performed under isocratic conditions with 75% solvent B and 25% solvent C at a flow rate of 0.2 mL / min.. The column is thermostrated at 40°C in a Shimadzu CTO-10AS column oven ('s-Hertogenbosch, The Netherlands). A Separations UV detector Model 757 (H.I. Ambacht, The Netherlands) (Fig. 2, detector 2) is used for detection of the enkephalins at 215 nm. Initial experiments were done using a Shimadzu autosampler, Model Sil-10A ('s-Hertogenbosch, The Netherlands), instead of using a loop. The injection volume was 5μL.

SEC-LC interface

The heart of the interface consists of two Rheodyne computer controlled six-port valves, Model Lab PRO (Cotati, CA, USA) with two connected sample loops. Each loop volume is 300 μL. A schematic diagram of the instrumental set up and the valve configurations are shown in Fig. 2. In this set-up, two valves are switched simultaneously, so there are only two possible configurations instead of four.

Procedures

In the first configuration of Fig. 2, after injection of a sample containing the model enkephalins and albumin, the effluent from the SEC-column is transported by pump 1 through detector 1 to loop 2. In the mean time pumps 2 and 3 transport solvent B *via* loop 1 onto the RP18-column. Effluent from the second column is always monitored by detector 2 . After switching to the second configuration the buffer in loop 1 is replaced by the effluent from the SEC column, while the content of loop 2 is transferred to the RPLC column, where separation of the enkephalins is performed.

The valves switch from the first to the second configuration at 3.80 min. after injection and the flushing of the RP-18 column is continued during 3 min., while the enkephalins are being

pre-concentrated. Subsequently, the composition of the mobile phase is changed to 75% solvent B and 25% solvent C (v/v). This is performed linearly in 20 s. Subsequently, using this mobile phase mixture, separation is performed, during 20 min. After elution, the composition of the mobile phase is changed back to solvent B in 20 s to obtain the initial conditions.

Test samples for the development of the chromatographic system

Development of the SEC is achieved with three samples, containing bovine albumin , a mixture of enkephalins and a mixture of enkephalins together with bovine albumin, respectively. Albumin is present in a concentration of 50 μg/mL, while the enkephalins are present in concentrations of 5 μg/mL .

Development of the RP-LC is performed with a sample, containing a mixture of enkephalins, dissolved in solvent B, with a concentration of 3 μg/mL.

Development of the interface is achieved with a sample containing a mixture of enkephalins and bovine albumin, dissolved in solvent A. All compounds are present in a concentration of 4 μg/mL, except for albumin (40 μg /mL) .

3. Results and discussion

Development of the SEC dimension

A SEC column with a pore size of 145 Å and a mobile phase with a composition, as described in the Experimental section, gave complete baseline resolution between albumin and the enkephalins at a flow rate of 0.2 mL/min., while the enkephalins were trapped in a single peak (Fig. 3). Under these conditions the retention time of albumin was 1.70 min whereas the retention time of the peak, containing the enkephalins was 2.75 min. The volume of the enkephalin peak was 200 – 250 μL. The temperature exhibited only minor effects on the k' values. An increase or decrease in temperature of 10^{o} C did not have a significant effect on the k' values. Lowering the pH of the mobile phase deteriorated the separation between albumin and the enkephalins. Increasing the pH did hardly affect the separation.

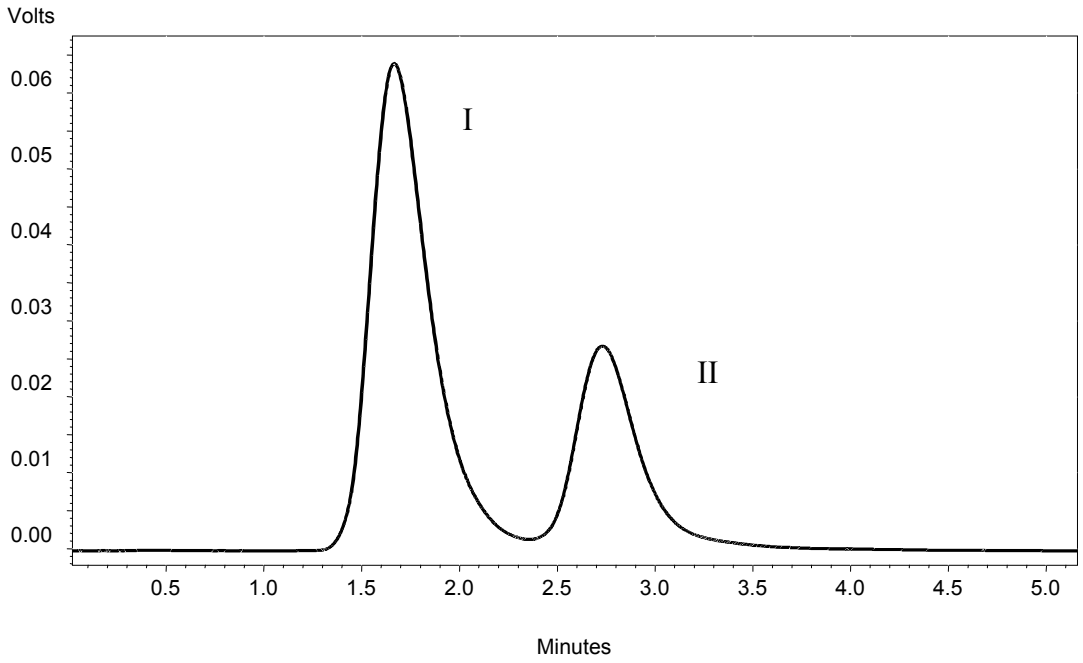

Fig.3. Separation between albumin (50 μg/mL) and the enkephalins (5 μg/mL each) I:albumin II: enkephalins.

Development of RP-LC dimension

For the separation of the enkephalins the parameters of the RP-LC dimension had to be made suitable by determining the effect of a change in pH and the amount of acetonitrile in the mobile phase on the k'-values of the enkephalins. The change in pH was performed by changing the contents of glacial acetic acid and ammonium acetate in solvents B and C, keeping the ratio of solvent B and solvent C in the mobile phase constant at 3:1 (v/v).

The change in the fraction of acetonitrile was performed by changing the amount of solvent C in the mobile phase. The results are given in Fig. 4 and 5. When the pH of the mobile phase exceeds 5, separation between the enkephalins becomes insufficient. Having free amino and carboxylic functions (Fig. 1), at a pH between 5 and 6.5, all enkephalins are in their zwitterionic form, in some cases exhibiting almost identical k'-values. However, at lower pH the retention of the enkephalins to the stationary phase increases, due to the formation of less polar, positively charged forms. Due to small differences between the pI-values of the employed enkephalins [28], the optimal pH for separation appears to be 4.8.

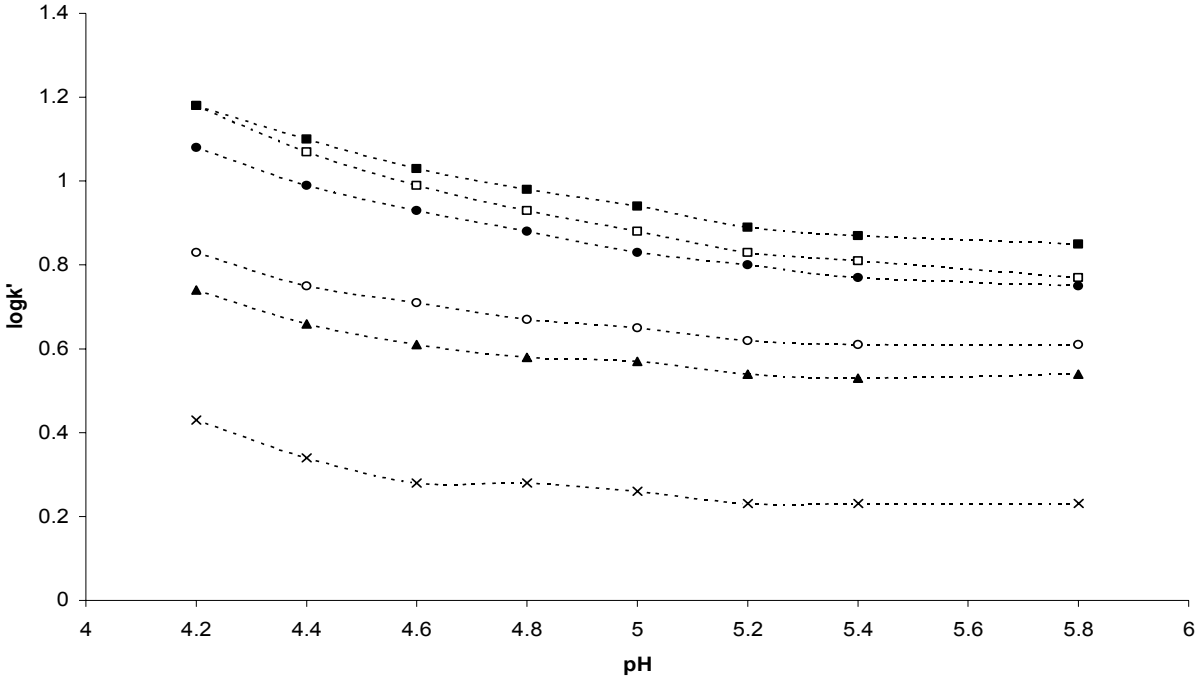

Fig. 4. Relation between log k' in RP-LC of the enkephalins and the pH. • leu-enkephalin, ▲met-enkephalin, □ des-tyr[D-ala^2-D-leu^5]-enkephalin, ×des-tyr-met-enkephalin, ■[ala^2]-leu-enkephalin, ○ [ala^2]-met-enkephalin.

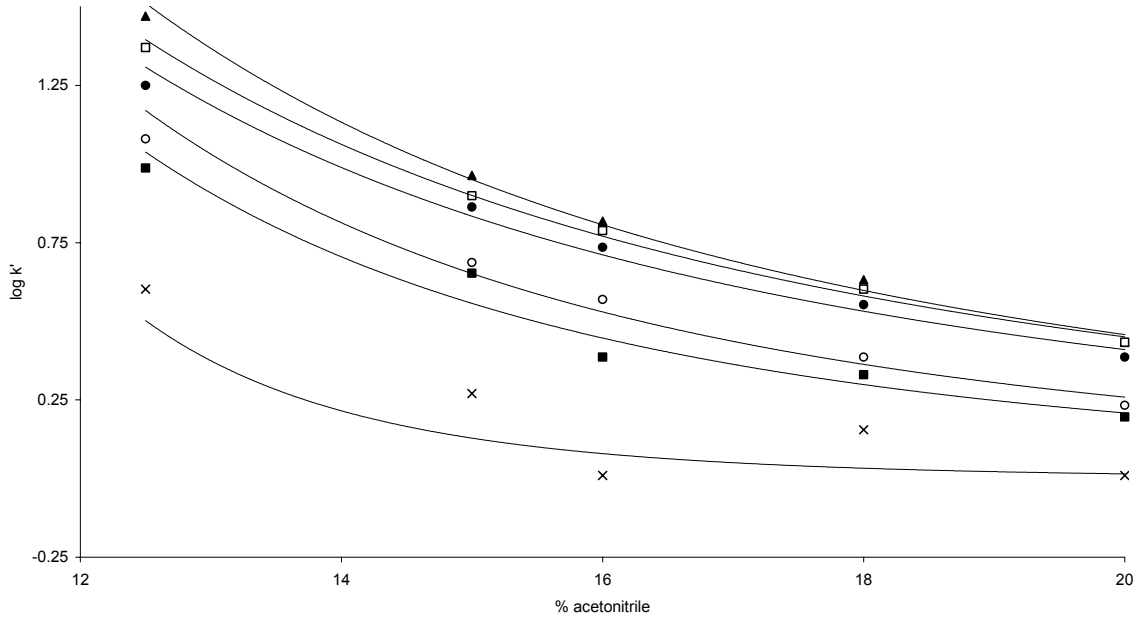

Fig. 5. Relation between the amount of acetonitrile and log k' in RP-LC of the enkephalins at pH 4.8. The lines are fitted according to [29], see text. •leu-enkephalin, ▲met-enkephalin, □des-tyr[D-ala^2-D-leu^5]-enkephalin, ×des-tyr-met-enkephalin, ■[ala^2]-leu-enkephalin, ○[ala^2]-met-enkephalin.

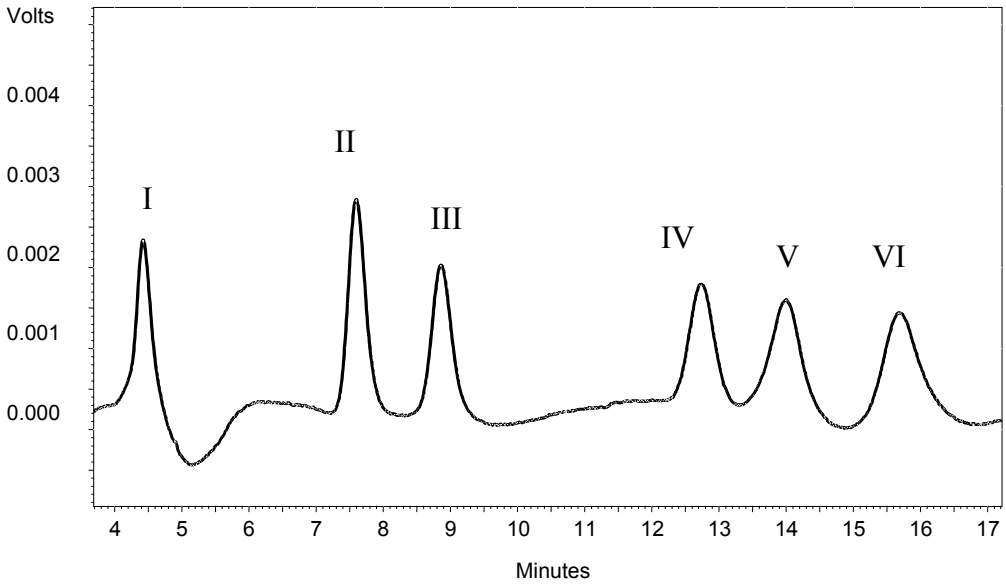

Fig. 6. Separation between the enkephalins (3 μg/mL each) in the RP-LC dimension. I: des-tyr-met-enkephalin. II: met-enkephalin. III: [ala^2]-met-enkephalin. IV: leu-enkephalin. V: des-tyr[D-ala^2-D-leu^5]-enkephalin. VI: [ala^2]-leu-enkephalin.

The lines in Fig. 5 are fitted according to the equation:

$$\text{Log } k' = A\Psi^2 + B\Psi + C \qquad [29]$$

This equation expresses the theoretical relationship between *k'* and the amount of modifier (Ψ). Lowering the acetonitrile content increases the difference in k' values, as shown in Fig. 5, resulting in the achievement of a better resolution between the peaks. However, below 15% acetonitrile, the retention times increase to a level where peak broadening causes insufficient separation of the compounds. Consequently, a better resolution may be achieved if both the column length and the amount of acetonitrile in the mobile phase will be decreased. Currently, a study on the miniaturization of the system, based on these findings, is in progress.

An illustration of the separation between the enkephalins at the conditions, as specified in the Experimental section, is given in Fig. 6.

Development of the coupled separation system

To minimize background signal of detector 2, as a result of the change in composition of the effluent after switching the valves, the solvents A, B and C have a similar content of buffer salts, so that minimal differences in refraction indices and UV light absorbing properties exist. For the same reason, the difference in flow in the two dimensions is kept minimal as

well. The effluent volume of the enkephalins at the first column is 200–250 μL, therefore a 300 μL loop was chosen to trap the complete enkephalins peak before injection onto the RP-LC column. After injection it takes 3.80 min to separate the enkephalins from albumin in the SEC and to fill the loop with the peptide fraction, resulting in a valve switch from the first to the second configuration at 3.80 min after injection. After that, theoretically, it takes 1.5 min. to empty the loop and bring the enkephalins onto the RP-LC column. Since concentration of the enkephalins on top of the separation column occurs, a flushing time of 3 min has been chosen.

If larger peptides have to be determined, it is possible to chose for a SEC-column packed with the same hydrophilic bonded silica, however with a different pore size. Consequently, the valves have to be switched at a different time.

An illustration of the separation between the enkephalins in the coupled system at the conditions, as specified in the experimental section, is given in Fig.7. From Fig. 6 an 7 the conclusion can be drawn that no detoriation of the enkephalin separation resulted from the coupling of the two dimensions whereas the level of preconcentration prior to RP-LC separation is high enough to diminish the peak broadening formed in the SEC separation completely. Fig. 6 and 7 also show that the rather broad peak shape from the slower eluting enkephalins is probably due to their long retention times. However, neither the separation nor the quantitation of the compounds (see below) is negatively influenced by these peak shapes.

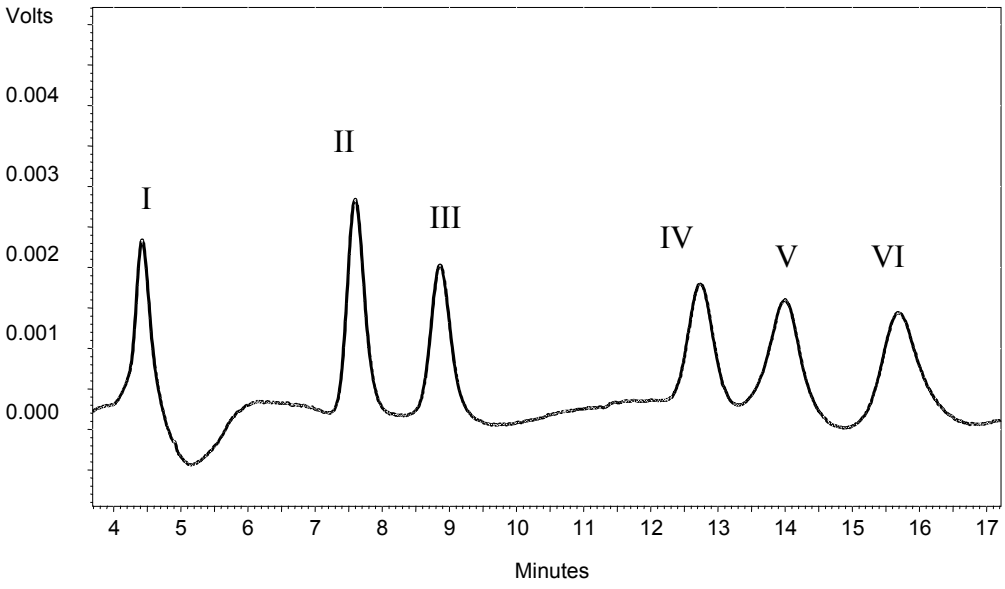

Fig. 7. Separation between the enkephalins (4 μg/mL each), obtained in the coupled system. I: des-tyr-met-enkephalin. II: met-enkephalin. III: [ala^2]-met-enkephalin. IV: leu-enkephalin. V: des-tyr[D-ala^2-D-leu^5]-enkephalin. VI: [ala^2]-leu-enkephalin.

Validation of the procedure

The procedure was validated for the *recovery* of the peptides, its *linearity* and *intra*- and *interday precision*.

The average *recovery* of the six enkephalins was determined at a low (7.5 ng) and a high (150 ng) amount. Recovery data were achieved by comparing the AUC of the enkephalins at detector 2 in the coupled system with the AUC at detector 2 after injection of the same amount of enkephalins directly onto the RP18-column. The recovery indicates the loss of enkephalins in both the SEC-column and the loop. The results are shown in Table 1.

Table 1 Recovery and relative standard deviation of several enkephalins in the coupled system

	7.5 ng injected Recovery ± RSD (%, n=5)	150 ng injected Recovery ± RSD (%, n=5)
met-enkephalin	91 ± 3.5	78 ± 2.0
leu-enkephalin	93 ± 5.5	81 ± 1.7
des-tyr[D-ala^2-D-leu^5]-enkephalin	83 ± 2.8	88 ± 7.1
[ala^2]-leu-enkephalin	85 ± 8.9	71 ± 0.9
[ala^2]-met-enkephalin	72 ± 1.2	66 ± 1.3
des-tyr-met-enkephalin	91 ± 4.4	88 ± 8.6

Generally, the average recovery of the samples containing 7.5 ng is higher than for the samples with 150 ng of enkephalins. Apparently, at higher concentrations the enkephalins are not completely trapped in the loop, due to a larger peak volume. To improve the recovery, enlarging of the sample loop may be a solution if samples, containing higher amounts of enkephalins have to be determined. Alternatively, miniaturization of the first dimension may lead to smaller peak volumes and, consequently, higher recoveries.

Linearity was determined over two decades in the presence of a constant amount of 200 ng albumin. The results are presented in Table 2. The correlation is good.

Table 2 Linearity: equation, standarddeviations and correlation

5-500 ng, n=24	Y = (a ± Sd a) X + b ± Sd b	R^2
Met-enkephalin	Y = (17037 ± 205) X - 11110 ± 8863	0.9983
leu-enkephalin	Y = (14069 ± 176) X + 4353 ± 7588	0.9982
des-tyr[D-ala^2-D-leu^5]-enkephalin	Y = (9307 ± 121) X + 4664 ± 5233	0.9981
[ala^2]-leu-enkephalin	Y = (11836 ± 240) X - 29544 ± 10357	0.9954
[ala^2]-met-enkephalin	Y = (16607 ± 181) X - 21253 ± 7831	0.9986
des-tyr-met-enkephalin	Y = (14134 ± 200) X - 3771 ± 8617	0.9978

Precision of the system was determined intraday and interday. This was performed with two different amounts of enkephalins. Again, the amount of albumine in the samples was 200 ng. The results are presented in Table 3. As expected, the average precision of the intraday assay is generally higher than the interday precision for both the high and the low ranges. Generally, the precision of the method is satisfactory.

Table 3 Intraday and interday precision of the developed system in the lower (10 ng) and the upper (500 ng) limit

	Interday 7.5 ng RSD (% , n = 5)	Intraday 7.5 ng RSD (% , n = 5)	Interday 500 ng RSD (% , n = 5)	Intraday 500 ng RSD (% , n = 5)
met-enkephalin	5.4	2.9	3.7	0.8
leu-enkephalin	6.0	8.1	2.6	1.2
des-tyr[D-ala^2-D-leu^5]-enkephalin	9.8	3.2	3.7	3.1
[ala^2]-leu-enkephalin	6.1	8.0	2.7	1.3
[ala^2]-met-enkephalin	3.5	2.4	2.2	1.6
des-tyr-met-enkephalin	4.9	2.4	0.9	1.0

The limit of detection (LOD) is 1.5 ng and is equal for all used enkephalins. This value was established, using a signal-to-noise ratio of 3. The limit of quantitation (LOQ) is also equal for all used enkephalins and determined to be 5 ng at a signal-to-noise ratio of 5. With the employed injection volume of 5 μL in the first dimension a concentration LOQ (CLOQ) of 1000 ng/mL is established.

4. Conclusion

The on-line coupled two-dimensional separation system, developed in this study, is suited for the quantitation of various enkephalins in the presence of albumin, with a satisfactory linearity, precision and recovery. This separation system was not yet tested for real biological samples, however, the removal of albumine using this method indicates that real biological samples should not be a problem. Studies to prove this are currently in progress. The level of preconcentration prior to RP-LC separation is high enough to reverse the peak broadening originated from the SEC separation completely. However, the method lacks the sensitivity to allow measurements of endogenous enkephalins in a biological matrix . The endogenous plasma concentration for leu-enkephalin and met-enkephalin is about 2 ng/mL [2], while the CLOQ is 1000 ng/mL. The CLOQ can be improved by using more sensitive detection methods. Another solution is the miniaturization of the coupled systems. Supposedly, peaks will become more sharp resulting in improved sensitivity of the method. The introduction of

an on-line concentration procedure may also contribute to a better sensitivity. Studies to improve the sensitivity of the method are currently in progress.

The employed heart-cut system proves to have a larger selectivity in comparison with a one-dimensional system, while the time of analysis is comparable. If more selectivity is desired, another dimension can be added. The added dimension has to be based on a different separation mechanism , *e.g.* capillary zone electrophoresis or mass spectrometry.

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Chapter 4

On-line coupling of size exclusion and reversed phase liquid chromatography for the analysis of structurally related enkephalins in cerebro-spinal fluid

Thom Stroink, Gerard Wiese[a], Jan Teeuwsen, Henk Lingeman, Joop C. M. Waterval, Auke Bult, Gerhardus J. de Jong, Willy J. M. Underberg

Abstract

On-line coupled analytical techniques can be advantageous in the assay of smaller peptides in complex biological matrices such as plasma, cerebro-spinal fluid and tissues.

The present study shows the feasibility of the recently reported on-line coupled size exclusion chromatography and reversed phase liquid chromatography separation system for the quantitation of structural related peptides in biological matrices, as demonstrated for a number of enkephalins in cerebro-spinal fluid. The degradation of the peptides, caused by endogenous peptidases in the matrix, could sufficiently be inhibited with imipramine HCL to allow an assay with satisfactory linearity and intraday (0.70-4.9%) precision. The sensitivity of the method, with a concentration limit of quantitation of 2 µg/mL is comparable with other kinds of assays for peptides and sufficient for the quantitation of peptide drugs with higher therapeutic ranges in biological matrices. However, for the assay of low concentrations of peptides, such as endogenous components of a biological matrix, the sensitivity may need improvement. The limit of quantitation cannot be improved by increasing the sample amount, because of interference of other endogenous components of the cerebro-spinal fluid. This indicates that a larger selectivity is desired. The LOQ may be improved by using more sensitive and selective detection methods such as mass spectrometry or fluorescence after post-column derivatization. Miniaturization of the system, combined with on-line trapping may also contribute to a better sensitivity.

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1. Introduction

Peptides are becoming more and more important as drug substances in the treatment of various diseases. Generally, these peptides are active in fairly low concentrations. Moreover, peptide profiling of biological samples gains a lot of attention nowadays, especially in proteomics research. The development of selective and sensitive methods for the analysis of these peptides has therefore become rather important. Selective analytical approaches involve the separation of the peptides under investigation from interfering compounds with structural similarity. As structural differences between peptides and proteins sometimes are very small, these separations may be tedious. The combination of two different separation principles has proven to be a powerful tool for the determination of peptide/protein mixtures [1-5].

Most of the analytical methods present for quantitation of peptides in biological matrices consist of a solid-phase sample pretreatment, followed by an analytical separation step [6-8] The application as a tool to combine on-line orthogonal separation mechanisms is less common. An increase in resolution and selectivity can be obtained only, when the coupled analytical dimensions are based on different separation mechanisms [9]. In addition of being orthogonal to preceding dimensions, a subsequent dimension should not reverse the resolution achieved by the previous ones [4, 9]. Therefore, coupled systems require an appropriate interface between the systems. In a coupled technique a loop can be used to trap the fraction of interest, whereafter a switching valve is employed to reinject this fraction. The on-line coupling of two separation systems for the analysis of peptides and proteins in a complex sample enables a significantly faster analysis in comparison with an off-line procedure. This because in off-line techniques transportation of the analytes from the first to the next dimension has to be performed manually [5]. Multidimensional on-line coupled analytical techniques can therefore be advantageous in the assay of smaller peptides in complex biological matrices such as plasma and tissues. If the separation of the complete sample in both dimensions is achieved, the on-line coupled system is considered to be completely multidimensional or comprehensive. This may be possible, if the time required for a separation on the second dimension will be the same as for filling a loop with effluent from the first dimension. A system that fulfills these requirements is described by Bushey and Jorgenson [4].

Another possibility is the use of a heart-cut system. In a heart-cut system only a fraction, retrieved from the first dimension, is brought into the second dimension where it is separated. The employment of coupled columns, including heart-cut and complete twodimensional

separation systems is not new. Column switching systems have been used for the determination of various compounds [10-17]. The heart-cut applications mainly exist of on-line sample pre-treatments, including sample enrichment for the quantitation of drugs, whereas the additional application as a tool to combine orthogonal separation mechanisms is less common. However, the complete two-dimensional separation systems applied for protein / peptide profiling, which combine mainly orthogonal separation mechanisms, *e.g.* the combination of a size exclusion chromatographic separation and a reversed phase chromatographic separation systems have not been employed for quantitative purposes [18-24].

Recently, a heart-cut coupled column system has been developed for the quantitation of structurally related peptides in complex matrices. This on-line coupled system uses size exclusion chromatography (SEC) and reversed phase liquid chromatography (RP-LC) in the separation and quantitation of a group of enkephalins in the presence of albumin [25]. The reported heart-cut system has sufficient selectivity, enabling quantitative determinations. However this system has not been applied for biological samples until now. The bioanalysis of enkephalins is important because the compounds play a role in the management of acute and chronic pain, which currently has a high priority. Enkephalins are neuropeptides with a morphinomimetical action and are mainly present and active in the brain. However, they are rapidly inactivated by several endogenous peptidases [26-28]. Currently, a lot of researchers investigate the possibility of in vivo inactivation of these enzymes to influence the intensity and length of their effects [26-29].

The present paper studies the feasibility of the recently reported on-line coupled two-dimensional separation system for the determination of structural related peptides in cerebro-spinal fluid (CSF), including the development of a sample pretreatment procedure for the inactivation of peptidases that may interfere in the results of the assay. As model compounds, again a number of enkephalins are chosen (fig. 1). Special attention has also been paid to the validation of the system.

2. Experimental

Chemicals

Glacial acetic acid, ammonium acetate and acetonitrile (analytical grade) were purchased from Merck (Darmstadt, Germany). Both the enkephalin-related peptides (leu-enkephalin, met-enkephalin, des-tyr[D-ala^2-D-leu^5]-enkephalin, des-tyr-met-enkephalin, [ala^2]-leu-

enkephalin, [ala^2]-met-enkephalin as well as imipramine HCl came from Sigma (St Louis, MO, USA). Cerebro-spinal fluid (CSF) was donated by the St. Anthonius Hospital (Nieuwegein, The Netherlands).

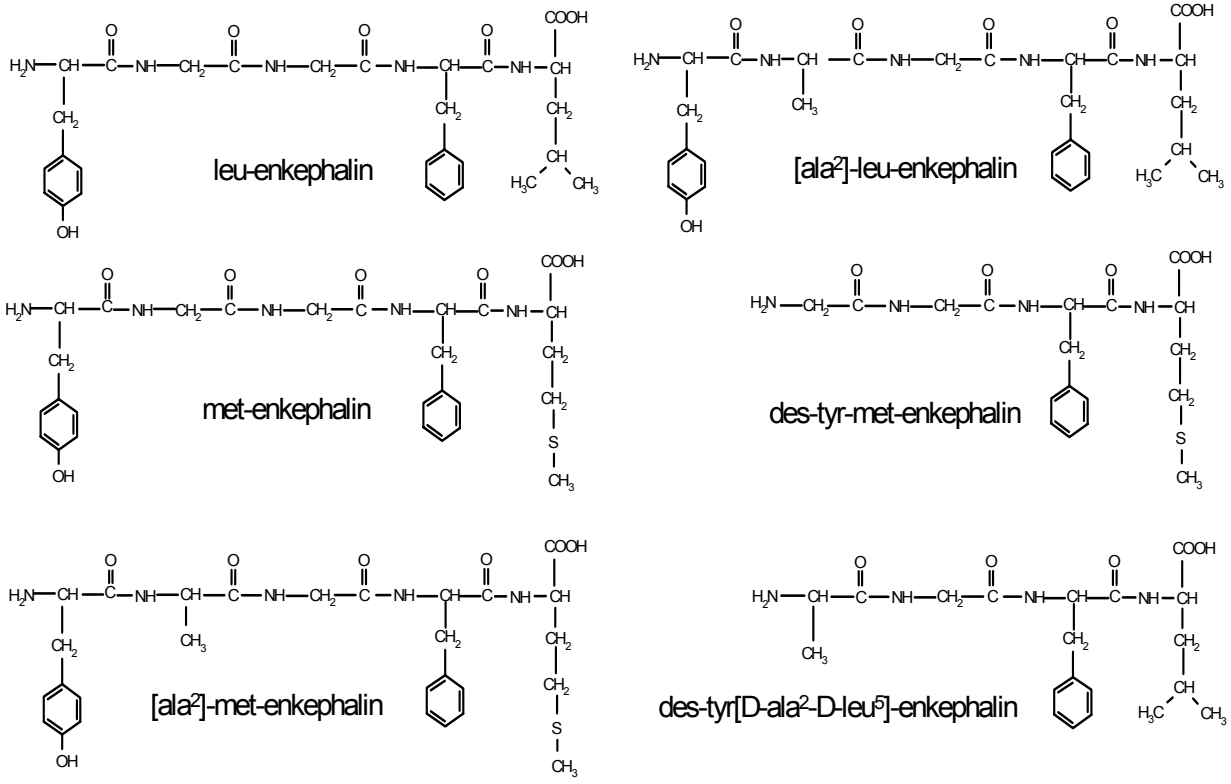

Fig. 1. Structures of the enkephalins.

Instrumentation, chromatographic conditions and procedures

The complete set-up of the system is given in Fig.2.

SEC-system

The first dimension (SEC) consists of a 4.6 mm i.d. × 30 mm stainless steel Phenomenex column packed with hydrophilically modified silica (Biosep SEC-2000©). The particle size is 5 µm and the pore size 145 Å (Bester, Amsterdam, The Netherlands). Both the exclusion and the inclusion volumes of the column are 0.2 mL. The column is thermostrated at 40°C in a Shimadzu CTO-10AS column oven (‘s-Hertogenbosch, The Netherlands). The mobile phase in this dimension (solvent A) is demineralized water with 0.01% w/w glacial acetic acid and

0.125 % w/w ammonium acetate (pH 5.8) and is degassed before use. The mobile phase is supplied by a Waters Associates Model M6000 pump (Milford, Massachusetts, USA) (Fig. 2, pump 1) at a flow rate of 0.2 mL / min. The detection is performed by a Shimadzu diode array detector Model SPD-M10 ('s-Hertogenbosch, The Netherlands) (Fig. 2, detector 1). The samples (5 µL) were injected onto the SEC column with a Shimadzu autosampler, Model Sil-10A ('s-Hertogenbosch, The Netherlands). Initial method development work was made with the SEC column outlet connected to a Separations UV detector Model 757 (H.I. Ambacht, The Netherlands). The wavelength is set at 215 nm.

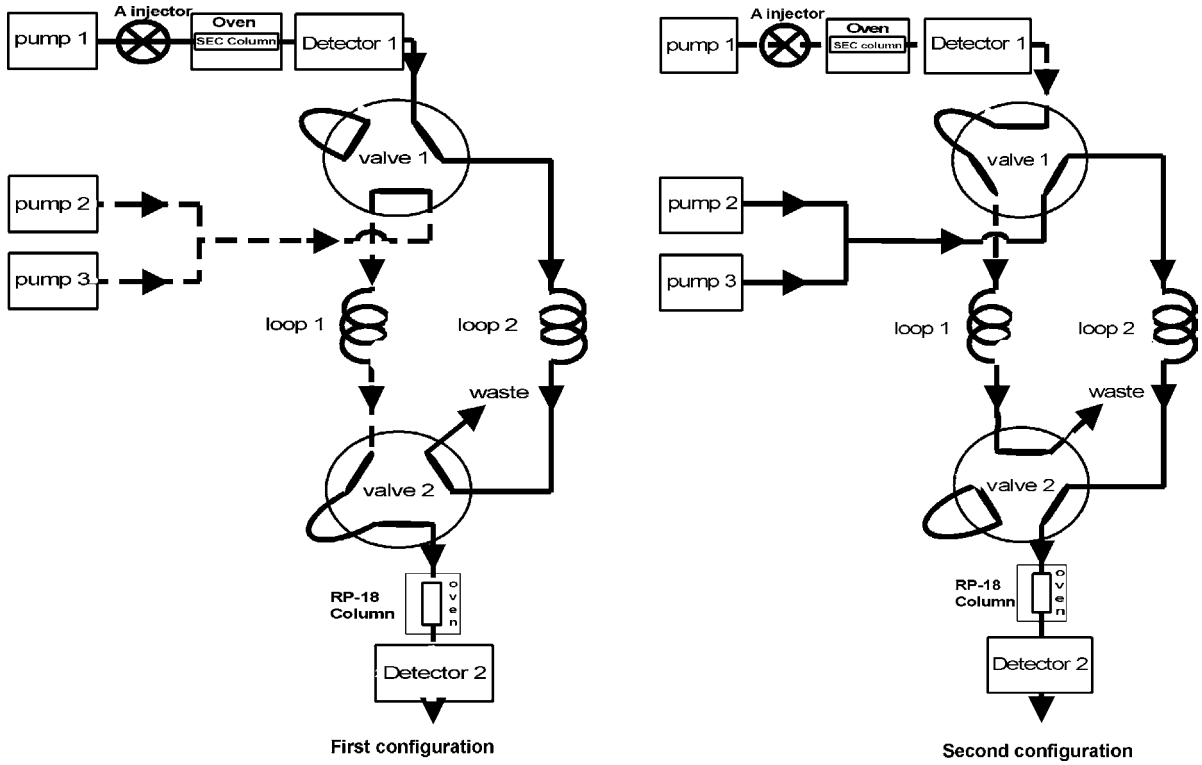

Fig. 2. Instrumental set up of the coupled SEC-LC system.

RP-LC-system

The second dimension (RP-LC) consists of a 2.0 mm i.d. × 125 mm stainless steel Phenomenex column, packed with C-18 bonded silica (Lichrosphere). The particle size is 3 µm and the pore size 100 Å (Bester, Amsterdam, The Netherlands). Chromatography is performed with a binairy system using two solvents. Solvent B is demineralized water with 0.05% w/w glacial acetic acid and 0.0625 % w/w ammonium acetate. The pH of solvent B is

4.8. Solvent C is solvent B to which 60% w/w acetonitrile is added. The solvents are degassed before use. The binairy system includes two pumps (Shimadzu SCL-10A pump,'s-Hertogenbosch, The Netherlands) (Fig. 2, pumps 2 and 3). The separation is performed under isocratic conditions with 75% solvent B and 25% solvent C at a flow rate of 0.2 mL / min.. The column is thermostrated at 40°C in a Shimadzu CTO-10AS column oven ('s-Hertogenbosch, The Netherlands). A Separations UV detector Model 757 (H.I. Ambacht, The Netherlands) (Fig. 2, detector 2) is used for detection of the enkephalins at 215 nm. Initial experiments were done using a Shimadzu autosampler, Model Sil-10A ('s-Hertogenbosch, The Netherlands), instead of a loop. The injection volume was 5 μL.

SEC-LC interface

The heart of the interface consists of two Rheodyne computer controlled six-port valves, Model Lab PRO (Cotati, CA, USA) with two connected sample loops. Each loop volume is 300 μL. A schematic diagram of the instrumental set up and the valve configurations are shown in Fig. 2. In this set-up, two valves are switched simultaneously, so there are only two possible configurations instead of four.

Procedures

In the first configuration of Fig. 2, after injection of a sample containing the model enkephalins and albumin, the effluent from the SEC-column is transported by pump 1 through detector 1 to loop 2. In the mean time pumps 2 and 3 transport solvent B *via* loop 1 onto the RP18-column. Effluent from the second column is always monitored by detector 2 . After switching to the second configuration the buffer in loop 1 is replaced by the effluent from the SEC column, while the content of loop 2 is transferred to the RPLC column, where separation of the enkephalins is performed.

The valves switch from the first to the second configuration at 3.80 min after injection and the flushing of the RP-18 column is continued during 3 min, while the enkephalins are being pre-concentrated at the top of the column. Subsequently, the composition of the mobile phase is changed to 75% solvent B and 25% solvent C (v/v). This is performed linearly in 20 s. Using this mobile phase mixture, separation is then performed, during 20 min After elution, the composition of the mobile phase is changed back to solvent B in 20 s to obtain the initial conditions.

Sample pretreatment

Optimization of the imipramine HCl concentration for the inactivation of the peptidases was performed using CSF samples, containing 10 µg/mL of each enkephalin and various concentrations of imipramine HCl: 0.01 M, 0.02 M and a blank. These samples were stored in the autosampler at 4° C.

Validation of the procedure was done with CSF samples containing the six enkephalins in various amounts, while the imipramine HCl concentration was always 0.02 M. The samples were stored at -18° C and analyzed at 4° C.

3. Results and discussion

In general, the performance of the system for the analysis of enkephalins in CSF is quite satisfactory. However, after 20-30 sample injections the system pressure tends to increase and a thorough flush of the injector and the loops with a SDS solution (1% w/w) is necessary to clean the system.

Sample stability

During the application of the system and the storage in the autosampler of CSF samples containing the enkephalins the degradation of the enkephalins showed not to be negligible (Table I). According to the literature the degradation of enkephalins is accelerated by various types and subtypes of peptidases. Today, it is still impossible to inhibit all of these enzymes completely [25-28]. However, a complete inhibition is not necessary. It is acceptable to work with a degradation rate that can be neglected during the course of the analysis in view of the precision of the analytical method. A degradation of about 2% in 2 hours seems acceptable, consequently, the half-life (t½) value has to exceed 70 hours.

Table I The relationship between the imipramine HCl concentration and the degradation rate, expressed by t½ in hours, of the employed enkephalins (10 µg/mL) in CSF samples

	Imipramine HCl concentration		
	0 M	0.01 M	0.02 M
met-enkephalin	173	173	990
leu-enkephalin	154	157.5	408
des-tyr[D-ala^2-D-leu^5]-enkephalin	315	693	770
[ala^2]-leu-enkephalin	49.5	59	173
[ala^2]-met-enkephalin	25.5	41	102
des-tyr-met-enkephalin	630	1155	1155

A short heat treatment of the CSF samples in general inactivates many enzymes, however, if the temperature becomes too high, denaturation of both the enzymes and other endogenous proteins may occur and, consequently, precipitation. The inhibiting effect of heat treatment on the enkephalin-degrading aminopeptidase activity in the CSF samples was determined by heating for 20 min at 70°. However, the aminopeptidase activity could not be sufficiently inhibited before denaturation of the proteins occurred.

Imipramine HCl is capable to in vivo inhibit enkephalin-degrading aminopeptidase activity in a concentration-dependent way [27]. The inhibiting effect of imipramine HCl on the peptidase-mediated degradation of the enkephalins was tested. The rate of decomposition was determined during storage of the CSF samples at 4° C for five days. Table I displays the influence of the concentration of imipramine HCl on the degradation rate, expressed by the half-life (t½) of the employed enkephalins in CSF samples. Based on these results a protective concentration of 0.02 M imipramine HCl was chosen. At this concentration the enkephalin degradation was negligible during the course of the analysis.

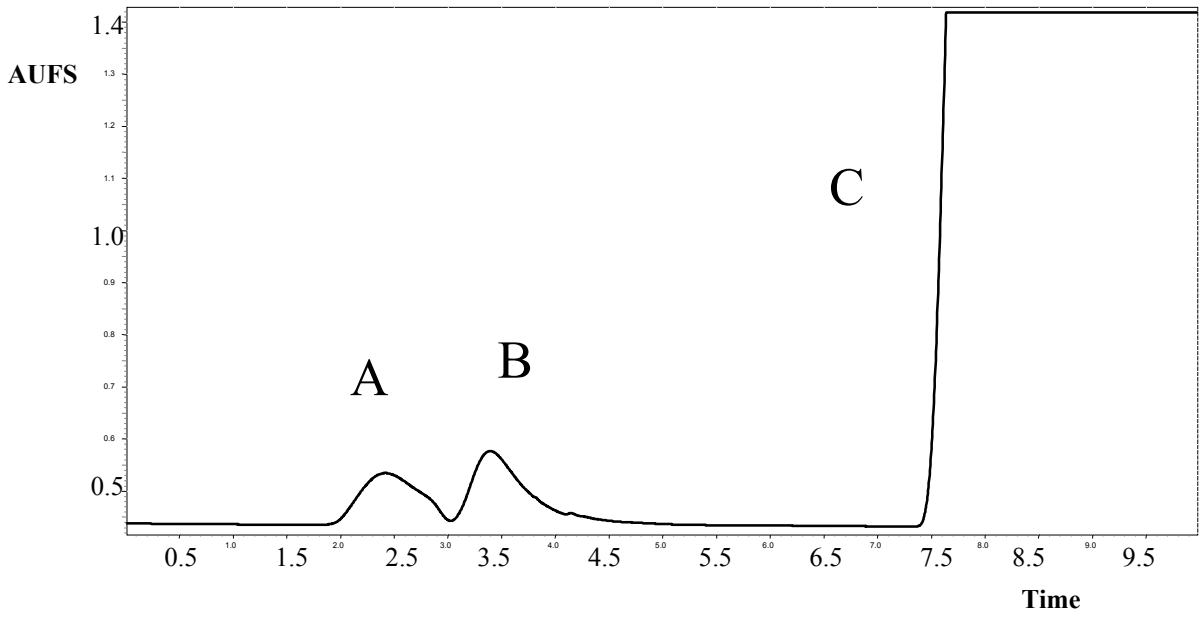

Fig. 3. Chromatogram of the SEC-separation. A: large proteins. B: small peptides (enkephalins). C: imipramine HCl.

Application of the separation system

Fig. 3 shows the SEC separation after injecting 10 μL of a CSF sample containing 100 ng of each enkephalin and 0.02 M imipramine HCl. In accordance with the previously reported

system [24] peak A represents large proteins such as albumin whereas peak B represents small peptides such as the enkephalins. Peak C consists of imipramine as was found by the analysis of three samples, one containing CSF only, one containing imipramine HCl only and another containing both imipramine HCl and CSF. However, the retention of imipramine can not be explained based on the SEC principle only, because peak A represents the exclusion volume ($V_{e\ (SEC)}$) while peak B represents the sum ($V_{(SEC)}$) of the exclusion ($V_{e\ (SEC)}$) and inclusion volumina ($V_{i\ (SEC)}$) of the SEC column. If pure SEC is valid, the retention volume can not exceed the sum of the exclusion and inclusion volumes. For peak C the following equation for the observed retention volume holds

$$V_{(observed)} = V_{(SEC)} + V_{(I)} + V_{(L)}$$

where $V_{(observed)}$ is the observed retention volume for peak C, $V_{(I)}$ the ionic interaction contribution and $V_{(L)}$ the ligand interaction contribution for a given solute [30]. The packing of the employed SEC-column consists of hydrophilically coated silica and is not further specified. Therefore the terms, $V_{(L)}$ and $V_{(i)}$ are unknown. However, in accordance with the equations, mentioned earlier, imipramine HCl seems to have a certain (electrostatic) interaction with the SEC-packing.

The separation between the enkephalins in the total system after injection of 10 μL of a CSF sample containing 5 μg/mL of each enkephalin is given in Fig. 4. There is no significant difference between the previously published figure of the RP-18 separation using SEC-RP-LC [24], implicating the efficient sample clean-up of the SEC dimension, including the removal of the large excess amount of imipramine HCL. Although the shape of the peak of des-tyr-met-enkephalin (Fig. 4, I) is influenced by the preceding negative signal it does not influence significantly the quantitation of this compound [24].

Validation of the procedure

The procedure was validated for its linearity and intra- and interday precision. *Linearity* was determined over about two decades (2-80 μg/mL). The results are presented in Table II. In general, the correlation is satisfactory. *Precision* of the system was determined intraday and interday by use of peak areas. This was performed with two different concentrations of enkephalins. The results are presented in Table III. As expected, the average precision of the intraday assay is generally better than the interday precision for both the higher (40 μg/mL) and the lower (20 μg/mL) concentration. For the determination of the enkephalins in CSF during several days and using one calibration curve, the interday precision is insufficient. The

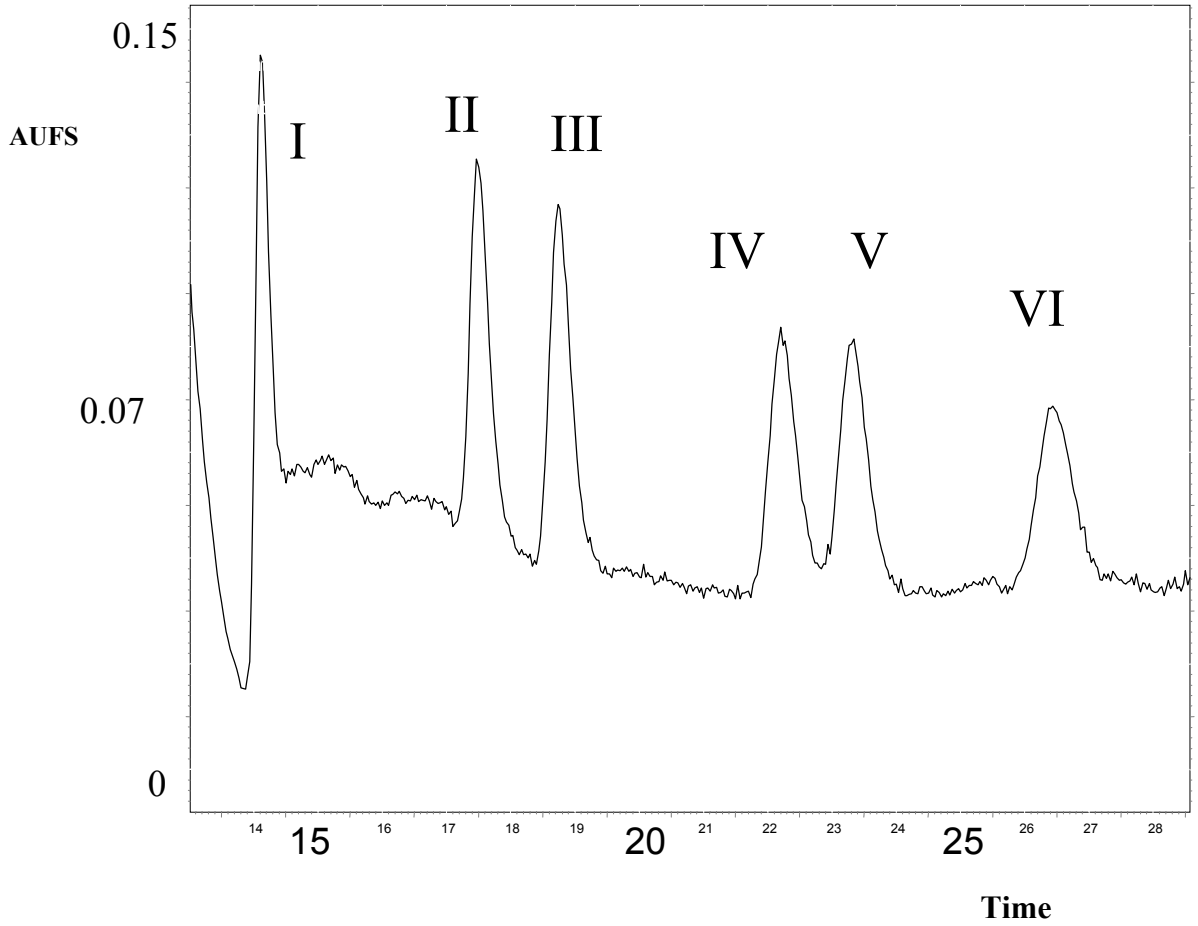

Fig.4. Chromatogram of the separation between the enkephalins (5 µg/mL each) after injection of 10 µL with the SEC - RP-LC system. I:des-tyr-met-enkephalin. II: met-enkephalin. III: [ala^2]-met-enkephalin. IV: leu-enkephalin. V: des-tyr[D-ala^2-D-leu^5]-enkephalin. VI: [ala^2]-leu-enkephalin.

Table II Linearity: equation, standard deviations and correlation coefficient

2-80 µg/mL, n=17	Y = (a ± Sd a) X + b ± Sd b	R^2
Met-enkephalin	Y = (31737 ± 828) X - 15010 ± 25555	0.99
leu-enkephalin	Y = (27330 ± 677) X - 1180 ± 20902	0.99
des-tyr[D-ala^2-D-leu^5]-enkephalin	Y = (28428 ± 1124) X - 51522 ± 346791	0.98
[ala^2]-leu-enkephalin	Y = (22911 ± 860) X + 18973 ± 26549	0.98
[ala^2]-met-enkephalin	Y = (34616 ± 1082) X - 30983 ± 33391	0.99
des-tyr-met-enkephalin	Y = (47145 ± 2595) X - 233766 ± 82513	0.96

Table III Intraday and interday precision of the system

	Interday 40 µg/mL RSD (% , n = 5)	Interday 20 µg/mL RSD (% , n = 5)	Intraday 40 µg/mL RSD (% , n = 5)	Intraday 20 µg/mL RSD (% , n = 5)
met-enkephalin	12	32	1.1	1.9
leu-enkephalin	17	25	0.90	3.2
des-tyr[D-ala^2-D-leu^5]-enkephalin	11	29	0.70	4.9
[ala^2]-leu-enkephalin	21	31	1.8	3.5
[ala^2]-met-enkephalin	8.3	32	1.5	4.6
des-tyr-met-enkephalin	5.9	19	2.9	2.7

higher amount can be determined more precisely than the lower amount. However, it is necessary to construct a calibration curve per day to obtain reliable results. The limit of detection (LOD) is 5 ng and is similar for all used enkephalins. This value was established, using a signal-to-noise ratio (S/N) of 3. Due to the interference of endogenous compounds in CSF near the detection limit, the limit of quantitation (LOQ) was turned out to be 20 ng, resulting in a concentration LOQ of 2 µg/mL. After injection of 20 µL of a CSF sample, spiked with enkephalins, serious interferences occured between the enkephalin peaks and endogenous components preventing reliable quantitation of the enkephalins. Consequently, the LOQ cannot be improved by increasing the injected sample volume.

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Chapter 5

On-line coupling of size exclusion chromatography and capillary zone electrophoresis *via* a reversed phase C18 trapping column for the determination of peptides in biological samples

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Abstract

Since biologically active peptides usually exhibit their effects in low concentrations, the development of sensitive analytical methods has become a challenge. In this paper a multi-dimensional system is presented, consisting of a size exclusion chromatographic (SEC) separation followed by a trapping procedure on a 4 mm x 3 mm i.d. reversed phase C18 (RP18) column with subsequent elution of the trapped fraction and separation by capillary zone electrophoresis (CZE). The system has been tested with mixtures of 6 enkephalins and albumin, mimicking biological matrices such as plasma and cerebro-spinal fluid. After separation of albumin from the enkephalins in the SEC dimension a heart-cut of 200 µL, containing the enkephalin peak, is taken, concentrated on the RP18 microcolumn and, after elution with a 20 µL plug of 80% acetonitrile, electrokinetically injected into the CZE system, where stacking and separation is achieved. While validation shows generally good linearity and reproducibility, quantitation limit with UV detection is acceptable (2.5 µg/ mL with an injection volume of 50 µL).

Electrophoresis 2003, 24, 1126-1134

1. Introduction

Peptide and protein assays in biological samples are increasingly important in the diagnosis and treatment of a number of diseases. Bioanalysis and pharmacokinetics of therapeutically used peptides as well as peptide profiles of biological samples in relation to diagnosis and treatment of diseases require suitable analytical methods for determination of these compounds. Since biologically active peptides usually exhibit their effects in fairly low concentrations, the development of sensitive analytical methods for this purpose is a challenge for many researchers. Selective analytical approaches involve the separation of the peptides under investigation from interfering compounds. As structural differences between peptides and proteins sometimes are very small, separations may be tedious. Most of the currently available analytical methods for quantitation of peptides in biological matrices consist of an off-line solid-phase sample pretreatment, followed by a separation step [1-3].

The on-line combination of two different separation principles has proven to be a powerful tool for the determination of peptide/protein mixtures [4-11]. In these cases, analyses are carried out in more than one analytical dimension in order to achieve the required resolution and/or selectivity [8-12]. An increase in resolution and selectivity can be obtained only when the various separation dimensions are orthogonal to each other [13]. However, a subsequent dimension should not reverse the resolution achieved by the previous ones [11, 13]. Coupled systems require appropriate interfaces between the dimensions. In a coupled system a direct transfer, a loop [10] or a trapping column [9] can be used to collect the fraction of interest, whereafter a switching valve or an elution procedure is employed to reinject this fraction. The on-line coupling of two separation systems for the quantitation of peptides in a complex sample enables a significantly faster analysis in comparison with an off-line solid-phase extraction, since in off-line techniques transportation of the analytes from the first to the next dimension has to be performed manually [12]. Multi-dimensional on-line coupled analytical techniques can therefore be advantageous in the assay of peptides in complex biological matrices such as plasma and tissues. Multi-dimensional separation systems can be divided into two classes: fully multi-dimensional or comprehensive systems and heart-cut systems.

If the separation of the complete sample in all dimensions is achieved, the on-line multi-dimensional system is considered to be comprehensive [11]. Another possibility is the use of a heart-cut system. A heart-cut system separates only a fraction, obtained from the first dimension, in the second dimension.

The employment of coupled columns, both heart-cut and complete two-dimensional separation systems, is not new. Column switching systems have been used for the determination of various peptides [14-15]. The combination of orthogonal separation mechanisms for heart-cutting in protein / peptide profiling is less common, in contrast to the complete two-dimensional separation systems [16-23]. However, these systems so far have rarely been employed for quantitative purposes [22,23]. A heart-cut system using SEC and reversed phase liquid chromatography (RP-LC) for quantitative purposes in peptide analysis was reported recently [24]. A disadvantage of RP-LC can be the use of denaturing modifiers, contrarily to CZE. The main advantage of CZE over RP-LC is the much higher separation efficiency. However, especially when trace level concentrations have to be determined, the analyte concentration detectability is often insufficient. Restrictions on the detector side can be overcome within certain limits. The use of mass spectrometric or laser induced fluorescence detection may increase the concentration sensitivity. However, the desired increase in concentration sensitivity primarily can be found by introducing analyte enrichment prior to CZE separation [25]. This can be achieved by introducing a trapping column. The preconcentration of an eluting heart-cut from the previous dimension on the trapping device can be performed via direct transport of a liquid stream to the CZE (on-line) or fully integrated in the CZE (in-line) [25].

The present study aims the development and validation of an on-line coupled multi-dimensional separation system, based on the heart-cut principle, using size exclusion chromatography (SEC) and concentration on a micro RP-LC column prior to CZE, to separate and quantitate a group of structurally related peptides in the presence of proteins such as albumin, which are the most interfering compounds in biological samples. Because of the better possibilities for quantitation of peptides in a complex matrix a heart-cut system is the best choice since it removes most of the interfering endogenous components of this matrix. Although the heart-cut principle is well known in bioanalysis, the application in peptide quantitation is rarely found in the literature. Only a few examples could be found, intended for peptide quantitation using two-dimensional LC-LC systems [14,15]. The application of the multi-dimensional SEC-CE system with a trapping column, as presented here, has not been described so far.

The validation is especially focused on the quantitative aspects of the system, whereas, in general, most existing LC-CE systems for peptide / protein analysis focus on detection and identification of the compounds only.

2. Materials and methods

2.1 Chemicals

Glacial acetic acid, ammonium acetate, phosphoric acid, sodium hydroxide and acetonitrile (analytical grade) were purchased from Merck (Darmstadt, Germany). The enkephalin peptides (Leu-enkephalin, Met-enkephalin, des-Tyr[D-Ala^2-D-Leu^5]-enkephalin, des-Tyr-Met-enkephalin, [Ala^2]-Leu-enkephalin, [Ala^2]-Met-enkephalin) came from Sigma (St Louis, MO, USA) as well as bovine albumin, purified by cold alcohol precipitation and essentially globulin free.

2.2 Instrumentation, chromatographic and electrophoretic conditions

The developed multi-dimensional separation system (Fig. 1) can be divided in various components: the SEC system, the reversed phase C18 (RP18) trapping system, the CZE interface and the CZE system. The components will be described separately.

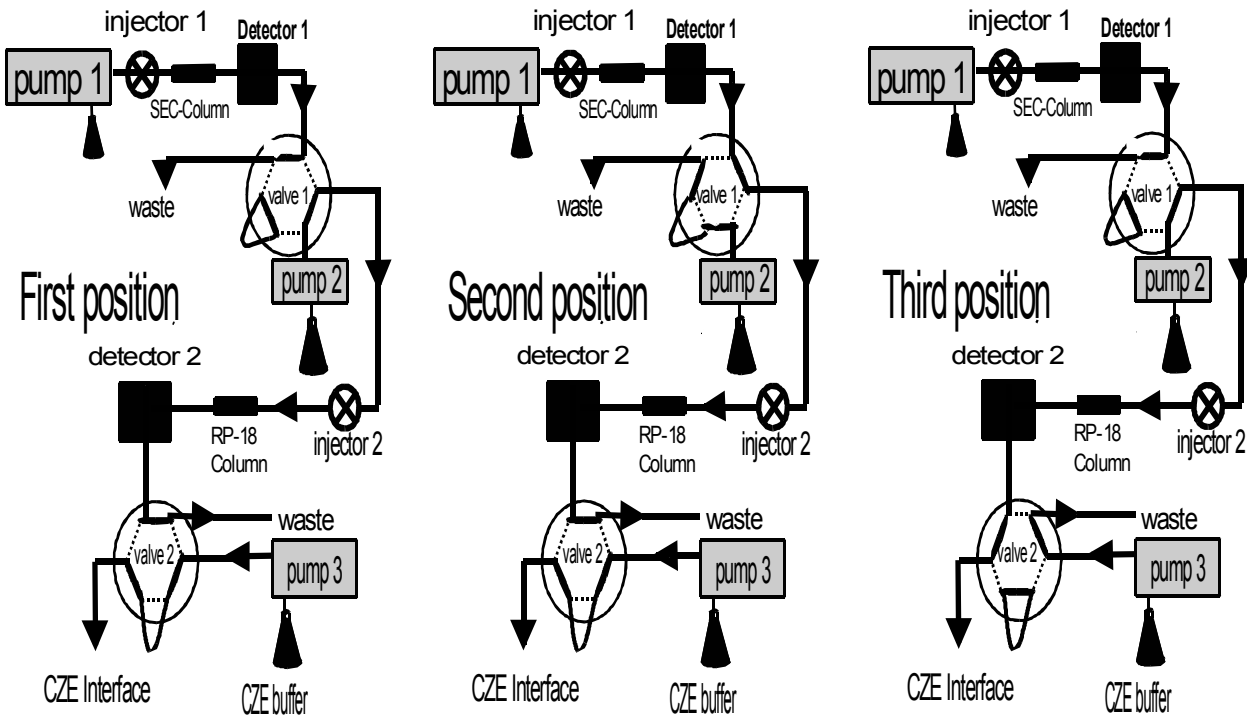

Fig. 1. Instrumental set up of the coupled system

2.2.1 Size exclusion chromatographic system

The first dimension (SEC) consists of a 4.6 mm i.d. × 30 mm stainless steel Phenomenex column packed with hydrophilically modified silica (Biosep SEC-2000©). The particle size is

5 μm and the pore size 145 Å (Bester, Amsterdam, The Netherlands). Both the exclusion and the inclusion volumina of the column are 200 μL. The column is thermostrated at 40°C in a Shimadzu CTO-10AS column oven (‘s-Hertogenbosch, The Netherlands). The mobile phase in this dimension (solvent A) is demineralized water containing 1.6 mM glacial acetic acid and 16 mM ammonium acetate (pH 5.8) and is ultrasonically degassed before use. The mobile phase is supplied by a Shimadzu SCL-10A pump (‘s-Hertogenbosch, The Netherlands) at a flow rate of 200 μL / min. (Fig. 1, pump 1) The detection is performed with an Applied Biosystems UV detector Model 757 (Separations, H.I. Ambacht, The Netherlands), set at a wavelength of 215 nm (Fig. 1, detector 1). The samples (50 μL) were injected onto the SEC column with a Waters U6K injector (Etten-Leur, The Netherlands) (Fig. 1, injector 1).

2.2.2 Reversed phase liquid chromatographic trapping system

This system consists of a 3.0 mm i.d. × 4 mm Securityguard^{tm} Phenomenex microcolumn, packed with C-18 bonded silica with a particle size of 5 μm (Bester, Amsterdam, The Netherlands). The column is connected to the previous SEC dimension with a Rheodyne six-port valve, model Lab PRO (Cotati, CA, USA) (Fig. 1, valve 1). The CZE interface (see Section 2.2.3) is connected to the trapping column with a similar valve (Fig. 1, valve 2). The mobile phase in the trapping system (solvent B) is demineralized water with 15 mM phosphoric acid adjusted to a pH 3.1 with 0.1 M NaOH and is filtered and degassed before use. The mobile phase is supplied by a Shimadzu SCL-10A pump (‘s-Hertogenbosch, The Netherlands) at a flow rate of 100 μL / min. (Fig. 1, pump 2). A Waters Associates U6K injector (Etten-Leur, The Netherlands) is placed before the micro column (Fig. 1, injector 2). Elution of the enkephalins is performed at ambient temperature using a 20 μL solvent plug containing 80% v/v acetonitrile in water. The detection is done with an Applied Biosystems UV detector Model 757 (Separations, H.I. Ambacht, The Netherlands), set at a wavelength of 215 nm (Fig. 1, detector 2).

2.2.3 Capillary zone electrophoretic interface

The home-made CZE interface (Fig. 2) is connected to valve 2 (Fig. 1) with stainless steel tubing (0.13 mm i.d., 1.59 mm (1/16") o.d.). The tubing supplies the effluent from the micro column or the CZE buffer. This buffer (solvent C) contains 150 mM phosphoric acid and is adjusted with 2 M NaOH to pH 3.1. Solvent C is supplied by a Waters Associates Model

M6000 pump (Etten-Leur, The Netherlands) (Fig. 1, pump 3) at a speed of 1000 μL/min. Simultaneously, the tubing serves as a grounded electrode. The interface is constructed from 20 mm of teflon (PTFE) tubing (1.52 mm (0.060") i.d., 3.18 mm (1/8") o.d.) At the bottom of this piece of PTFE tubing the stainless steel tubing is inserted for 4 mm and at the top a piece of PTFE tubing (20 mm, 0.38 mm (0.015") i.d., 1.59 mm (1/16") o.d.) is inserted for 10 mm, resulting in an interface compartment with a volume of 11 μL. On top of the interface a device is placed to collect the sample excess and put it to the waste (Fig.2). The CZE capillary is inserted in the PTFE tubing to a distance of 2 mm from the steel tubing, as indicated in Fig. 2.

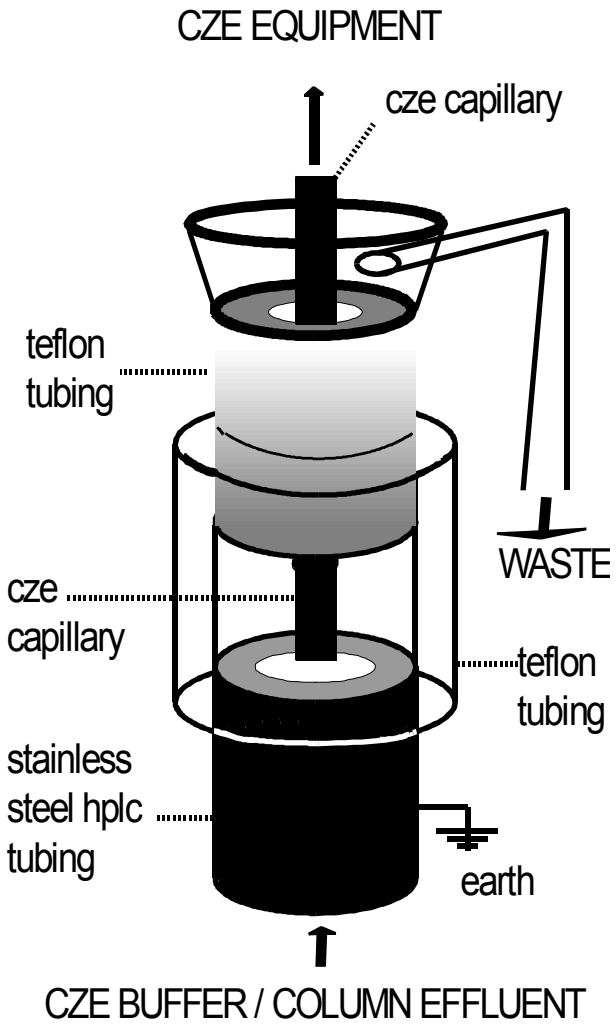

Fig. 2. Schematic representation of the developed LC-CZE interface

2.2.4 Capillary zone electrophoretic system

A PrinCE capillary electrophoretic system (PrinCE Technologies, Emmen, The Netherlands) is used. The capillaries are cut from a batch of fused silica capillaries (50 μm I.D., 375 μm O.D.), purchased from Bester B.V. (Amstelveen, The Netherlands) A total length of 90 cm is employed and a detector window is made at 45 cm from the inlet side of the capillary. The one lift mode of the PrinCE system is used. A potential of -30 kV is applied to the outlet side of the capillary, while the inlet side is grounded. Detection is performed with an Applied Biosystems 785 A Absorbance Detector (Separations, H.I. Ambacht, The Netherlands), adjusted for use in CZE and set at a wavelength of 215 nm.

2.3 Procedures

In the first position of Fig. 1, a sample containing the model peptides and albumin are injected (t_0). Subsequently, separation between the peptides and albumin is performed and the albumine fraction is transported to the waste. At 2.25 min after t_0, valve 1 is switched to the second position. The effluent containing the peptides is transported by pump 1 through injector 2 to the RP18 trapping column. In the mean time pump 3 transports the CZE buffer (solvent C) through the CZE interface. At 3.25 min after t_0 valve 1 is switched again to the first position and pump 2 purges solvent B through the trapping column *via* detector 2 to the waste to remove the ammonium acetate. At 9.50 min after t_0 a 20 μL plug of 80% acetonitrile in water is injected *via* injector 2 and the effluent from the RP18 column is monitored by detector 2 . Valve 2 is switched at 10.50 min after t_0, while the voltage (-30 kV) of the CZE apparatus is applied with a ramp of 3 kV/s. At this point the third position is achieved. Subsequently, solvent B, containing the elution plug, is transported to the CZE interface, where electrokinetic injection of the peptides from the interface into the CZE is performed. Subsequently, valve 2 is switched at 11.25 min after t_0, thus re-establishing the first position of the system, after which the CZE separation of the enkephalins is completed.

3. Results and discussion

As model peptides a group of enkephalins has been chosen. Fig. 3 shows their structures and illustrates their degree of similarity. Despite minor differences in structure these peptides all have approximately the same size. Therefore, the choice of a SEC column serving as first dimension is logical, since, using the heart-cut principle, all peptides of interest have to be

transported to the next dimension simultaneously. A disadvantage of the chosen SEC system is the limited separation efficiency resulting in larger peak volumes. However, this is of minor importance when it is used as a first dimension before heart-cut with subsequent preconcentration. The RP18 trapping device is chosen to desalt the SEC effluent and to concentrate the sample prior to CZE injection to improve the inherently low concentration sensitivity of CZE.

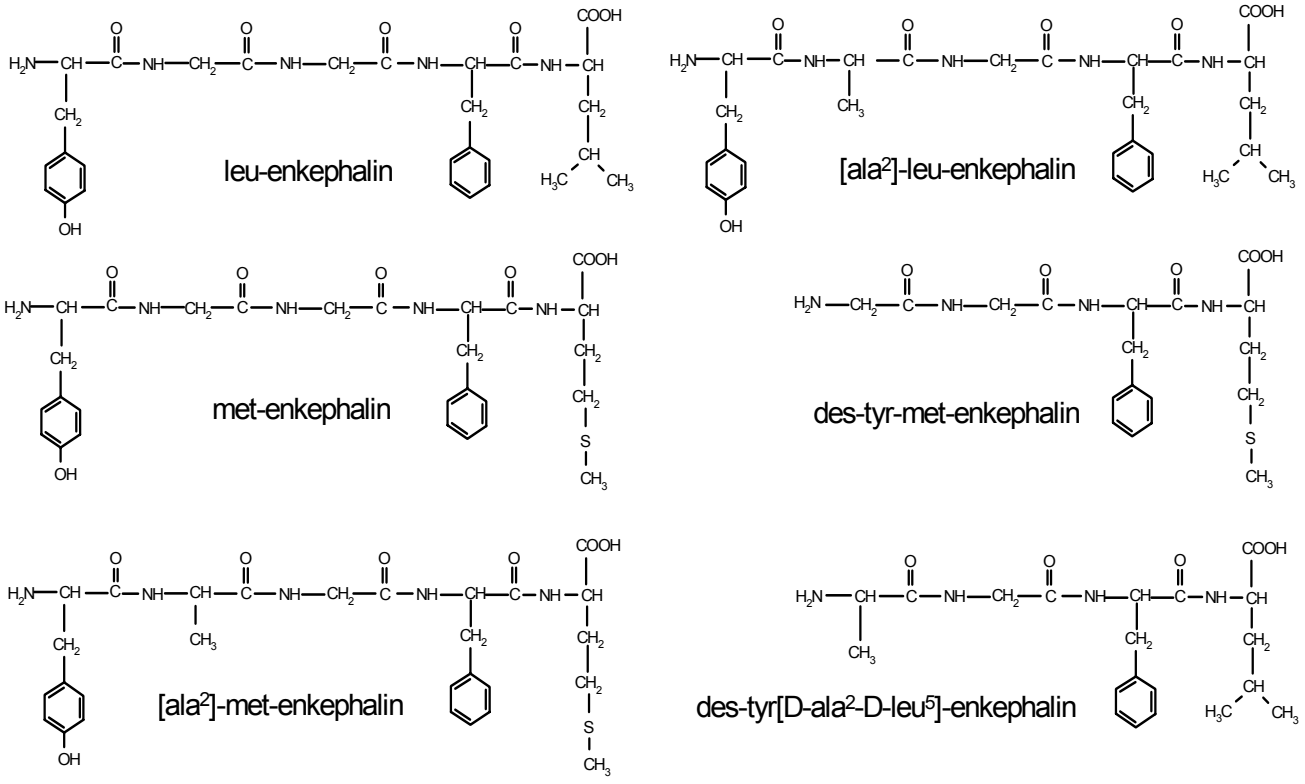

Fig. 3. Structures of the enkephalins

The development of the multi-dimensional separation system can be divided in various components: the SEC system, the RP18 trapping system, the CZE interface and the CZE system. These components have to be optimized separately. However, during the development the compatibility of the separate components to each other has to be kept in mind. Each component will be discussed and focus will be laid on its development and its compatibility with the total system.

3.1 Development of the size exclusion chromatographic system

SEC was chosen as the first dimension, since the peptides of interest are much smaller in size than interfering proteins such as albumin. The SEC column has to separate the peptides from albumin. A SEC column with a pore size of 145 Å and a mobile phase of demineralized water containing 1.6 mM glacial acetic acid and 16 mM ammonium acetate (pH 5.8) gave complete baseline resolution between albumin and the peptides at a flow rate of 200 µL/min, while the peptides were eluted in a single peak with a volume of around 250 µL. The temperature exhibited only minor effects. An increase or decrease in temperature of 10^{o} C did not have a significant effect on retention and resolution. Exceeding the sample volume over 50 µL or lowering the pH of the mobile phase deteriorated the separation between albumin and the peptides. Increasing the pH did hardly affect the separation. Fig. 4 illustrates the separation between albumin and the peptides after a 50 µL injection of a sample containing 4 µg/mL albumin and 2.5 µg/mL of each peptide.

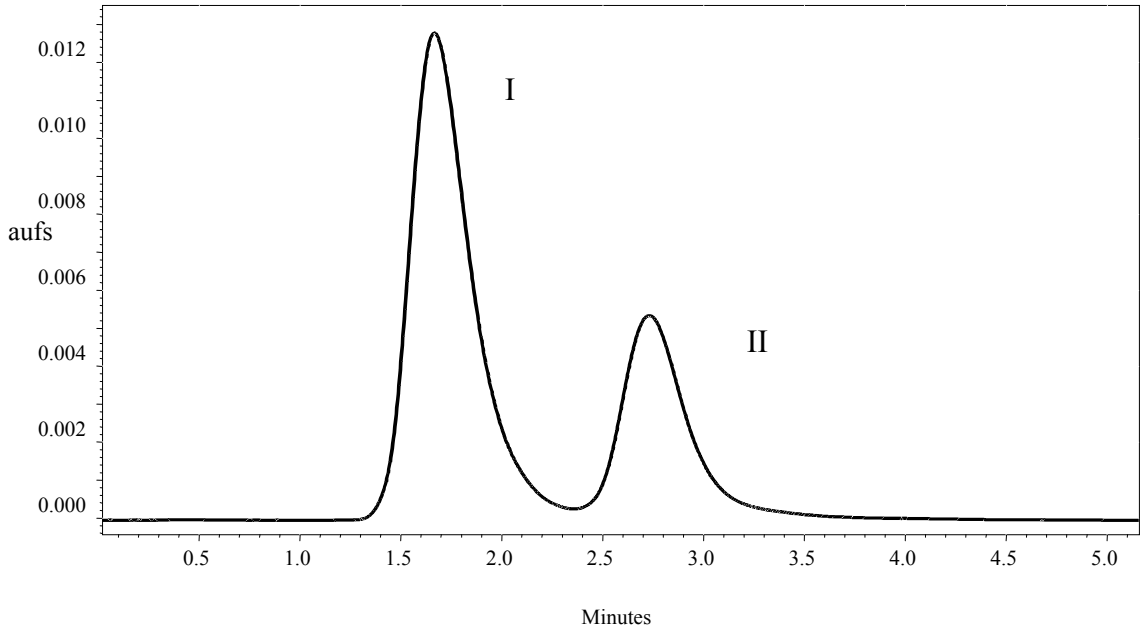

Fig. 4. Separation between albumin (4 µg/mL) and six enkephalins (2.5 µg/mL each) in the SEC dimension I: albumin II: enkephalins

3.2 Development of the trapping system

The RP18 trapping device has to remove the ammonium acetate from the peptide fraction eluting from the SEC column and also concentrate the sample. There are four main requirements for the RP18 trapping system:

(i) The breakthrough volumes of the peptides on the trapping device have to be known and have to exceed 300 µL significantly (250 µL for the peak volume of the SEC column and an additional volume for tubing and washing). (ii) The buffer (solvent B) of the trapping system has to be compatible with the CZE buffer (solvent C) but the electrical conductivity has to be lower than the CZE buffer to allow the enlargement of the injection by introducing stacking conditions. (iii) It has to be possible to desorb the peptides simultaneously using a desorption volume which is as small as possible in order to maintain a high overall preconcentration factor. (iiii) The flow of the buffer and, consequently, the passage time of the eluting plug through the CZE interface has to be compatible with the maximum injectable amount in the CZE system. These four items will be discussed separately.

Ad (i). The breakthrough volumes of [Ala^2]-Leu-enkephalin and [Ala^2]-Met-enkephalin were determined, according to the procedure described by Werkhoven-Groewie et al. [26], on three micro RP18 columns, one with a dimension of 3.0 mm i.d. × 4 mm, another with a dimension of 0.8 mm i.d. × 2 mm and a third one with a dimension of 0.3 mm i.d. × 1 mm. First, the micro columns were washed with solvent B. Subsequently the columns were loaded with solvent B containing 1 µg/mL of [Ala^2]-Leu-enkephalin or [Ala^2]-Met-enkephalin and the UV signal monitored at 215 nm. The breakthrough volumes on the two smallest columns of 0.8 mm i.d. × 2 mm and 0.3 mm i.d. ×1 mm were about 100 µL and 10 µL, respectively. Only the breakthrough volumes on the column of 3.0 mm i.d. × 4 mm sufficiently exceeded the necessary 300 µL. For [Ala^2]-Leu-enkephalin the average volume was 0.82 ± 0.09 mL (n = 5) and for [Ala^2]-Met-enkephalin 0.81 ± 0.08 mL (n = 5). Since all model peptides have a similar structure, it can be assumed that the breakthrough volumes of the other compounds will be similar.

Ad (ii). In the CZE system the best separation between the enkephalins occurred using a separation buffer of 150 mM phosphate adjusted to a pH of 3.1 (see Section 3.4). Solvent B contains the same buffer, adjusted to the same pH, and is, therefore, compatible with the CZE buffer. However, the phosphate concentration has been decreased to 15 mM phosphate to allow optimum stacking conditions. When the concentration was further decreased, the electrical resistance in the CZE interface increased, with a greater possibility for a selective

electrokinetic injection from the CZE interface into the CZE, which has to be avoided (see Section 3.3, ad (i)).

Ad (iii). As desorption solvent a mixture of 80% (v/v) acetonitrile in water was selected. This solvent is suitable for desorption of peptides and commonly used for that purpose [27]. The acetonitrile-water ratio was not varied, but the amount was optimized. The minimum volume to get maximum desorption was 20 μL. The average recovery for the desorption of [Ala^2]-Leu-enkephalin and [Ala^2]-Met-enkephalin, was determined with a low (100 ng) and a high (1000 ng) amount. The recovery is determined using an additional RP-LC column coupled to the trapping column instead of the CZE interface. This RP-LC dimension for the quantitation of enkephalins is previously described [24]. Peak areas of the model compounds determined after injection using injector 2, trapping, elution and separation on the RP-LC were compared with areas achieved after direct injections onto the RP-LC column. The recovery was better than 90 %, indicating that loss of the peptides during elution was acceptable.

Ad (iiii). The influence of the flow during desorption of the peptides (1 μg of each) on the peak broadening of the desorbed peak was determined after elution with a 20 μL solvent plug containing 80% v/v acetonitrile in water using detector 2 (Fig. 1). A flow of 400 μL/min resulted in a minimum peak volume of 50 μL and, consequently, maximum preconcentration, while both increase and decrease of the flow enlarged the peak volume. However, at a flow of 400 μL/min the passage of the elution plug through the CZE interface takes 0.125 min, while the maximum injection time is 0.5 min. This means that at a flow of 400 μL/min only a small amount will be injected, resulting in a loss in sensitivity. The optimum flow was 100 μL/min, resulting in a peak volume of 100 μL. With an injection time of 0.5 min, half of the peak can be transported through the CZE interface. The best results can be expected when using the middle part of the peak, containing the highest concentrations of the peptides. If longer injection times are used, resolution in the CZE dimension was decreased in an unacceptable way.

3.3 Development of the capillary zone electrophoretic interface

Only amounts of solvent in the nL region can be injected into the CZE, while generally amounts of effluent of 100 μL and more, containing the analytes of interest, are available for injection after elution from a small LC column. An additional problem is the flow difference between the CZE and LC systems. The CZE interface has to overcome these problems. The CZE interface should allow the injection of a reproducible amount eluting from the trapping column as well.

There are two main requirements for the CZE interface: (i) It is necessary to have a non-selective injection of the peptides to avoid discrimination in sensitivity. (ii) The interface has to supply CZE buffer, free from gas bubbles, to the inlet of the capillary during the CZE run. Only a small part of the effluent from the trapping column can be injected, therefore the interface has to serve during injection as a waste as well.

Ad (i). Using CZE, hydrodynamic injection enables a non-selective injection, while electrokinetic injection in general is more selective. However, hydrodynamic injection is complicated to use after on-line coupling because it is necessary to optimize exactly the timing of the applied pressure for injection. This may be overcome by injecting during 30 sec at a very low pressure. However, the lower the applied pressure the lower the reproducibility of the injection amount. Consequently, a procedure for an electrokinetic injection with minimum selectivity was developed. In general, the length L of the sample zone using electrokinetic injection is given by

$$L = (V_{ep} + V_{eo})\, t_{inj} \qquad \text{(Eq. 1)}$$

where V_{ep} is the electrophoretic velocity of the sample molecule, V_{eo} is the electroosmotic velocity of the sample solution and t_{inj} is the injection time [28]. To determine the influence of the terms V_{ep} and V_{eo} , the electrical resistance in the interface between the tubing, which was connected to earth and served as electrode, and the inlet of the capillary was measured. The resistance during a CZE run was about 25 Ω, while during injection it was about 50 Ω. Both during the run and during injection the applied voltage was –30 kV, while the current was around 100 μA, indicating a total system resistance of 300 MΩ. Consequently, since the resistance in the interface is negligible compared to the total system resistance, there is no additional electric field in the interface. This means that analytes, both cations and anions, only move into the capillary by electroosmosis. During injection and CZE run the electroosmotic flow has to be constant. This is indicated by the fact that the peak heights and migration times must be constant during analysis. During the various injections and runs, the peak heights and migration times indeed showed only small variations indicating a constant electroosmotic flow.

Ad (ii). During elution from the trapping column, about 50 μL is transported through the interface compartment. Consequently, the volume of the interface compartment can not be too large to avoid peak broadening. The volume chosen is 11 μL. If the volume was made smaller, the formation of gas bubbles during the CZE run was observed, resulting in current break downs, unless the supply of CZE buffer was increased by increasing the flow of pump

3 (Fig 1). However, the flow of the CZE buffer through the interface is limited by its dimensions. If the flow becomes too high, the pressure in the compartment will increase and CZE buffer will be forced through the interface by pressure, which is unfavourable. Using the interface with the dimensions, mentioned in the materials and methods section, the maximum flow is 1000 µL/min. At this rate the pressure in the interface is negligible and the electroosmotic flow is not influenced. An additional advantage of a relatively fast CZE buffer supply is the quick removal of the effluent from the trapping column still present in the tubing between valve 2 and the interface (Fig. 1) after switching valve 2 to the initial position and electrokinetic injection.

3.4 Development of the capillary zone electrophoretic system

The aim of the CZE dimension is the separation between the enkephalin peptides to allow quantitation. During development, various parameters involved in CZE separations were investigated to get the required separation, resulting in the CZE system as described in the materials and methods section. The most important parameters, the pH of the running buffer and the buffer ionic strength, will here be discussed briefly.

The optimum pH range for the CZE separation was determined by calculating charge-to-mass vs pH curves for the enkephalins, using the pKa values of their acidic and basic functions. According to these curves the largest differences in charge-to-mass ratios between the enkephalins were observed between pH 2.0 and pH 3.5. All peptides have a net positive charge in this range. Other studies indicate good separations in the same pH range [29-30] This cannot be explained by these differences only, since the ionic strength of the solution and the nature of the buffer matrix also have an (unpredictable) influence [29]. The resolution between the enkephalins was determined, using various types of separation buffers and with several pH values within the range of 2.0 – 3.5. The best results were achieved using a 150 mM phosphate buffer with a pH of 3.1. If phosphate buffers with higher concentration were used the current became higher, resulting in the formation of gas bubbles in the interface. Lower concentrations resulted in less stacking and, consequently, decreased separation efficiency. Fig. 5 illustrates the separation between the enkephalins in the CZE dimension after injection of 50 µL of a sample containing 4 µg/mL albumin and 10 µg/mL of each enkephalin, using the complete system as described in the materials and methods section.

3.5 Injection efficiency

The average recovery from the applied SEC dimension is 80%, as was reported earlier. [24]. The recovery from the trapping column is slightly higher (about 90 %, see Section 3.2). Consequently, the amount of peptides present in the peak eluting from the trapping column will be about 70 % of the initially injected amount, while only half of this peak is available for electrokinetic injection (see Section 3.2). However, that part of the peak is chosen which contains the highest amount of peptides. By comparing peak heights achieved after CZE separations with hydrodynamic injections and peak heights using the complete system an estimate was made about the real amount of the peptides, detected in the CZE dimension. About 1-2% w/w of the initially injected amount could be finally detected in the CZE dimension.

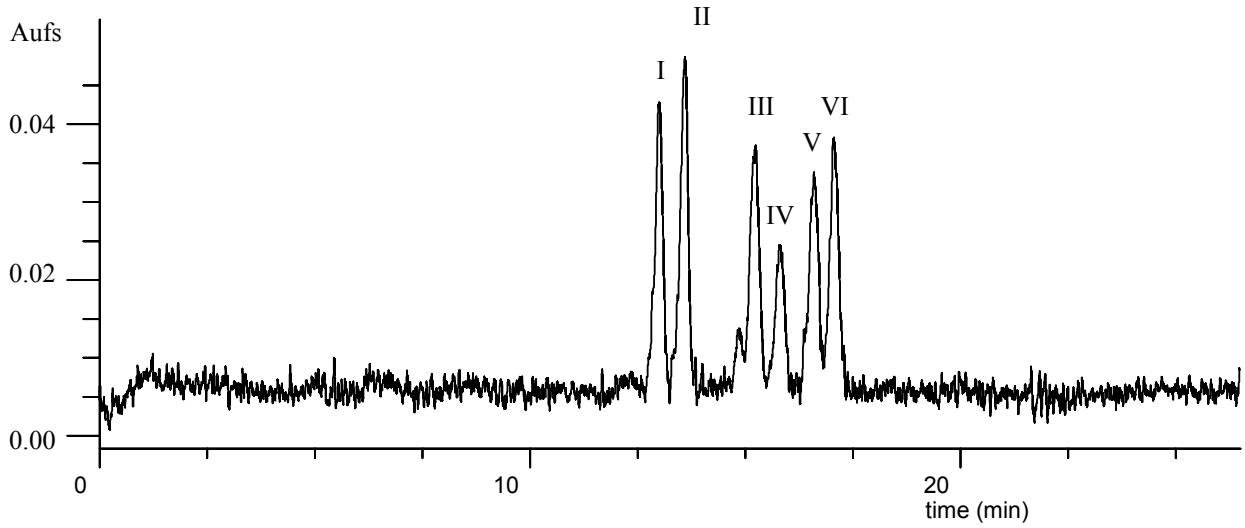

Fig. 5. Electropherogram of six enkephalins after injection of 50 µL of a sample containing 4 µg/mL albumin and 10 µg/mL of each enkephalin, using the complete system as described in the materials and methods section. I: des-Tyr[D-Ala^2-D-Leu^5]-enkephalin, II: des-Tyr-Met-enkephalin, III: [Ala^2]-Leu-enkephalin, IV: Leu-enkephalin, V: [Ala^2]-Met-enkephalin, VI: Met-enkephalin

3.6 Validation of the procedure

The procedure was validated for its linearity and intra- and interday precision. *Linearity* was determined, using peak heights, over about two decades (2.5-100 µg/mL). The results are

presented in Table I. The correlation is satisfactory. *Precision* of the system was determined intraday and interday using peak heights as well. This was performed for the interday experiment with two different concentrations of enkephalins, 5 μg/mL and 20 μg/mL, while the intraday precision was determined using a concentration of 10 μg/mL The results are presented in Table II. As expected, the average precision of the intraday assay is much higher than the interday precision with both the higher (20 μg/mL) and the lower (5 μg/mL) concentration. The limit of detection (LOD) is 75 ng and is similar for all used enkephalin peptides. This value was established, using a signal to noise ratio (S/N) of 3. The limit of quantitation (LOQ) is about 125 ng, equal for all used enkephalins at a S/N of 5. Consequently, with the employed injection volume of 50 μL the concentration LOQ (CLOQ) is 2.5 μg/mL.

Table I
Linearity: equation, standard deviations and correlation. Y, a, Sd, X and b represents respectively peakheight, slope of the line, standard deviation, concentration (μg/mL) of the enkephalin and the intercept.

2.5 –100 μg/mL, n=30	Y = (a ± Sd a) X + b ± Sd b	R^2
Met-enkephalin	Y = (0.296 ± 0.0069) X + 0.656 ± 0.28	0.99
Leu-enkephalin	Y = (0.117 ± 0.0035) X + 0.575 ± 0.14	0.99
des-Tyr[D-Ala^2-D-Leu^5]-enkephalin	Y = (0.137 ± 0.0052) X + 979 ± 0.21	0.98
[Ala^2]-Leu-enkephalin	Y = (0.168 ± 0.0056) X + 1.01 ± 0.18	0.99
[Ala^2]-Met-enkephalin	Y = (0.210 ± 0.0063) X + 0.649 ± 0.25	0.99
des-Tyr-Met-enkephalin	Y = (0.285 ± 0.0054) X + 0.255 ± 0.22	0.99

Table II
Intraday and interday precision of the system

	Interday 5 μg/mL RSD (% , n = 5)	Interday 20 μg/mL RSD (% , n = 5)	Intraday 10 μg/mL RSD (% , n = 5)
Met-enkephalin	7.5	12.6	4.2
Leu-enkephalin	16.4	6.9	2.8
des-Tyr[D-Ala^2-D-Leu^5]-enkephalin	16.1	9.8	4.2
[Ala^2]-Leu-enkephalin	12.9	9.5	4.7
[Ala^2]-Met-enkephalin	15.9	10.8	11
des-Tyr-Met-enkephalin	13.2	12.4	7.1

4. Conclusion and future perspectives

The on-line coupled multi-dimensional separation system, developed in this study, is suitable for the quantitation of various enkephalins in the presence of a similar concentration of albumin with a satisfactory linearity and intraday precision. The removal of albumine

indicates that the analysis of real biological samples will be possible with this system although the concentration of large proteins mostly is higher in these samples. Systematic studies to prove this are currently in progress. As an example, Fig. 6 presents an electropherogram of a cerebro-spinal fluid sample spiked with six enkephalins in a concentration of 40 µg/mL each. Fig. 6 shows, that it is possible to analyze these peptides in biological matrices such as cerebro-spinal fluid, indicating the larger selectivity of this multi-dimensional system in comparison with an one-dimensional system, while the time of analysis is comparable. The differences in migration times of the peptides between Fig. 5 and 6, although in both cases the times have minor differences during various runs, may be due to alterations at the capillary wall, due to endogenous components from the cerebro-spinal fluid.

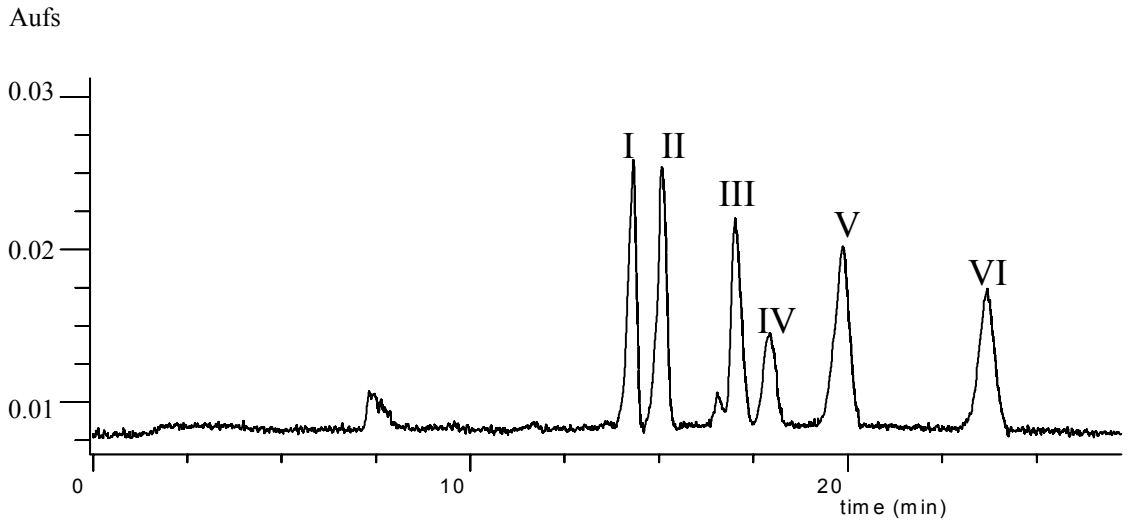

Fig. 6. Electropherogram of six enkephalins after injection of 10 µL of a cerebro-spinal fluid sample spiked with 40 µg/mL of each enkephalin, using the complete system as described in the materials and methods section. I: des-Tyr[D-Ala^2-D-Leu^5]-enkephalin, II: des-Tyr-Met-enkephalin, III: [Ala^2]-Leu-enkephalin, IV: Leu-enkephalin, V: [Ala^2]-Met-enkephalin, VI: Met-enkephalin

However, the level of preconcentration prior to CZE separation is limited. The CLOQ of this system is 2.5 µg/mL, which is typical for separation systems based on electrophoresis. However, biologically active peptides may be present in lower concentrations in body fluids. If necessary**,** the CLOQ may be improved by using more sensitive detection methods. The replacement of the 50 µm i.d. capillary for a 75 µm i.d. one may increase the sensitivity, supposedly up to five times [31]. However, in this case, the current will increase, resulting in

lower separation efficiency and, therefore, resolution. Another solution may be the miniaturization of the SEC and the trapping column. The development of an additional in-line CZE preconcentration procedure may also contribute to a better sensitivity [25]. However, this will result in a more complex system. Studies to improve the sensitivity of the method are currently in progress. If more selectivity is desired, another dimension can be added. The added dimension has to be based on a different principle, *e.g.*mass spectrometry. This may have the advantage of increased sensitivity as well.

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Chapter 6

On-line coupling of size exclusion and capillary zone electrophoresis via a reversed phase C18 trapping column for the analysis of structurally related enkephalins in cerebro-spinal fluid

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Abstract

On-line coupled analytical techniques can be advantageous in the assay of smaller peptides in complex biological matrices such as plasma, cerebro-spinal fluid (CSF) and tissues. The present study shows the feasibility of a recently developed system, consisting of a size exclusion chromatographic (SEC) separation followed by a trapping procedure on a RP18 microcolumn with subsequent elution of the trapped fraction and separation by capillary zone electrophoresis (CZE) for the quantitation of structural related peptides in biological matrices, as demonstrated for a number of enkephalins in CSF. After SEC separation of the enkephalins from large proteins present in CSF a heart-cut of 200 µL, containing the enkephalin peak, is taken, concentrated on the RP18 microcolumn and, after elution of the enkephalins with 80% acetonitrile, a fration of the eluate is electrokinetically injected into the CZE system, where stacking and separation is achieved**.** The degradation of the peptides, caused by endogenous peptidases in the matrix, is sufficiently inhibited with imipramine HCl. The assay has a satisfactory linearity and intraday (9.70-16,3%) precision considering the complexity of this multi-dimensional separation system. The sensitivity of the method, with a concentration limit of quantitation of 2.5 µg/mL is comparable with other CZE assays for peptides and sufficient for the quantitation of peptide drugs in biological matrices.

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1. Introduction

Peptides gain more and more importance as drug substances in the treatment of various diseases. Commonly, these peptides are active in fairly low concentrations. Further, peptide profiling of biological samples gains a lot of attention nowadays, especially in proteomics research. This requires the development of selective and sensitive methods for the analysis of these peptides. Selective analytical approaches include the separation of the peptides under investigation from interfering compounds with structural similarity. As structural differences between peptides and proteins sometimes are very small, these separations may be tedious. The coupling of two different separation principles has proven to be a strong tool for the determination of peptide/protein mixtures [1-5]. Nearly all of the analytical methods for quantitation of peptides in biological matrices consist of an off-line solid-phase sample pretreatment, followed by an analytical separation step [6-8]. On-line coupling of orthogonal separation systems can increase the selectivity and sensitivity of the analysis in a reduced amount of time [5]. An increase in resolution and selectivity can be obtained only, when the coupled analytical dimensions are based on different separation mechanisms [9], while a subsequent dimension does not reverse the resolution achieved by the previous ones [4, 9]. Moreover, coupled systems require a suitable interface between the dimensions. In a coupled system a column or a loop can be used to trap the fraction of interest, whereafter a switching valve is employed to reinject this fraction. The employment of coupled columns, both heart-cut and complete two-dimensional separation systems, is not new. Column switching systems have been used for the determination of various peptides and proteins [10-17]. However, these systems have only been employed for qualitative purposes [18-24].

Recently, a heart-cut multi-dimensional size exclusion chromatography (SEC)-reversed phase liquid chromatography (RP-LC) system for the quantitation of structurally related peptides in complex matrices has been reported [25]. With this on-line coupled system the separation and quantitation of a group of enkephalins in cerebro-spinal fluid (CSF) could be achieved with a limit of quantitation (LOQ) of 20 ng at an employed injection volume of 10 μL. This LOQ could not be improved by increasing the injected sample volume due to the interference of endogenous compounds in CSF near the detection limit. Another recently reported system, consisting of a SEC separation followed by a trapping procedure on a 4 mm x 3 mm i.d. reversed phase C18 (RP18) column with subsequent elution of the trapped fraction and separation by capillary zone electrophoresis (CZE), for the quantitation of structural related peptides is challenging as well [26]. This system presumably posseses a larger separation

efficiency as demonstrated here with the application to the determination of enkephalins in CSF.

The bioanalysis of enkephalins is important because these compounds play a role in the control of acute and chronic pain, which currently has a high priority. Enkephalins are neuropeptides with a morphinomimetical action and are mainly present and active in the brain. However, they are rapidly inactivated by several endogenous peptidases [27-28]. Currently, a lot of researchers investigate the possibility of in vivo inactivation of these enzymes to influence the intensity and length of their effects [27-29].

The present paper shows the feasibility of the reported SEC-CZE system for the quantitation of structural related peptides in biological matrices, as demonstrated for a number of enkephalins (Fig. 1) in CSF. Special attention has also been paid to the validation of the system.

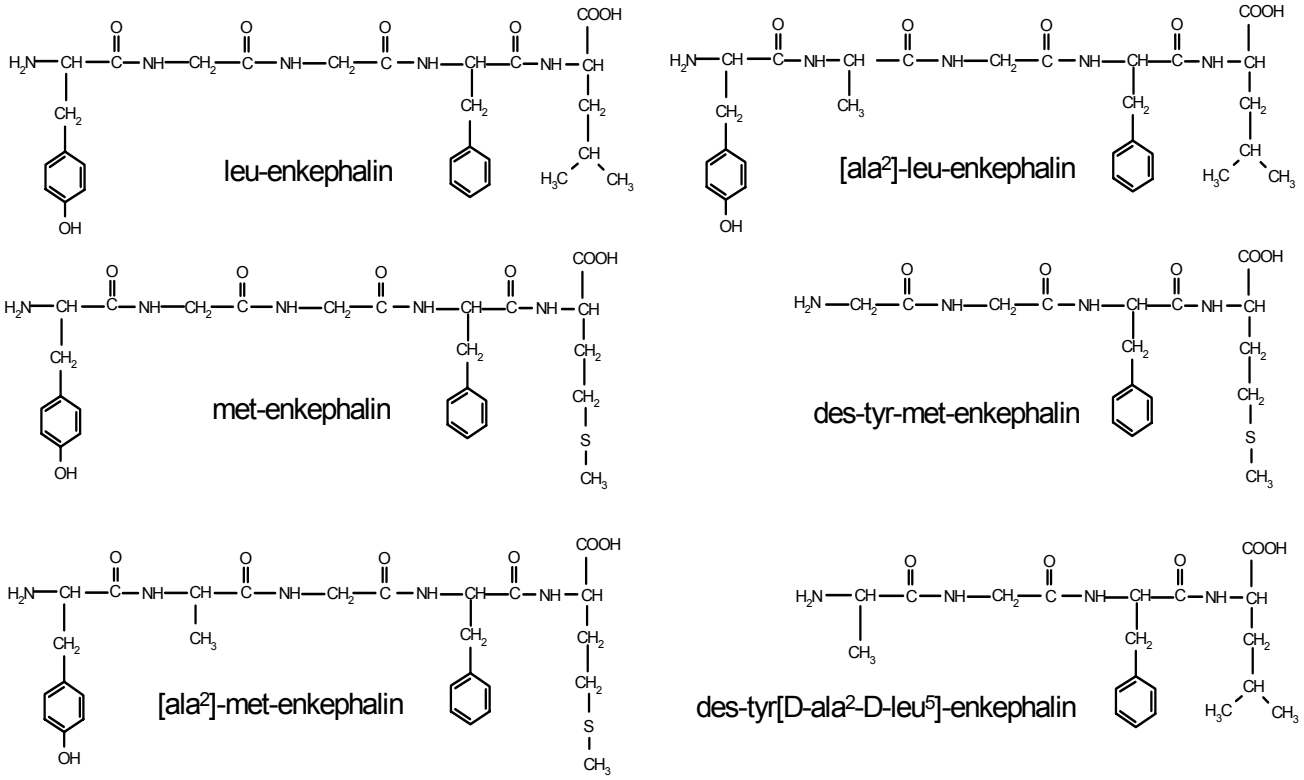

Fig. 1. Structures of the enkephalins

2. Material and methods

2.1 Chemicals

Glacial acetic acid, ammonium acetate, phosphoric acid, sodium hydroxide and acetonitrile (analytical grade) were purchased from Merck (Darmstadt, Germany). The enkephalin peptides (Leu-enkephalin, Met-enkephalin, des-Tyr[D-Ala^2-D-Leu^5]-enkephalin, des-Tyr-Met-enkephalin, [Ala^2]-Leu-enkephalin, [Ala^2]-Met-enkephalin) came from Sigma (St Louis, MO, USA). Cerebro-spinal fluid (CSF) was donated by the St. Anthonius Hospital (Nieuwegein, The Netherlands).

2.2 Instrumentation, chromatographic and electrophoretic conditions

The developed multi-dimensional separation system, shown in Fig. 2, can be divided in various components: the SEC system, the reversed phase C18 (RP18) trapping system, the CZE interface and the CZE system. The components will be described separately.

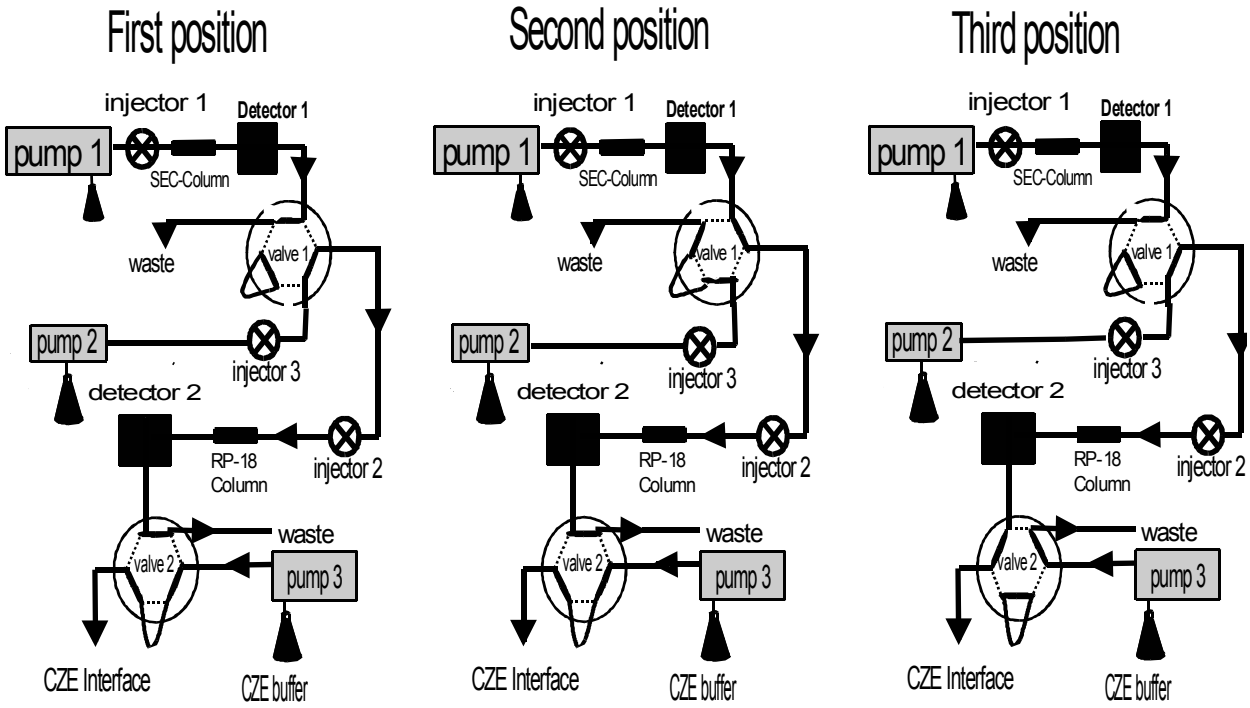

Fig. 2. Instrumental set up of the coupled system

2.2.1 Size exclusion chromatographic system

The first dimension (SEC) consists of a 4.6 mm i.d. × 30 mm stainless steel Phenomenex column packed with hydrophilically modified silica (Biosep SEC-2000©). The particle size is 5 μm and the pore size 145 Å (Bester, Amsterdam, The Netherlands). Both the exclusion and the inclusion volumina of the column are 200 μL. The column is thermostrated at 40°C in a Shimadzu CTO-10AS column oven ('s-Hertogenbosch, The Netherlands). The mobile phase in this dimension (solvent A) is demineralized water containing 1.6 mM glacial acetic acid and 16 mM ammonium acetate (pH 5.8) and is ultrasonically degassed before use. The mobile phase is supplied by a Shimadzu SCL-10A pump ('s-Hertogenbosch, The Netherlands) at a flow rate of 200 μL / min. (Fig. 2, pump 1) The detection is performed with an Applied Biosystems UV detector Model 757 (Separations, H.I. Ambacht, The Netherlands), set at a wavelength of 215 nm (Fig. 2, detector 1). The samples (10 μL) were injected onto the SEC column with a Waters U6K injector (Etten-Leur, The Netherlands) (Fig.2, injector 1).

2.2.2 Reversed phase liquid chromatographic trapping system

The column is a 3.0 mm i.d. × 4 mm Securityguard™ Phenomenex microcolumn, packed with C-18 bonded silica with a particle size of 5 μm (Bester, Amsterdam, The Netherlands). The column is connected to the previous SEC dimension with a Rheodyne six-port valve, model Lab PRO (Cotati, CA, USA) (Fig. 2, valve 1). The CZE interface (see Section 2.2.3) is connected to the other side of the trapping column with a similar valve (Fig. 2, valve 2). The mobile phase in the trapping system (solvent B) is demineralized water with 15 mM phosphoric acid adjusted to a pH 3.1 with 0.1 M NaOH and is filtered and degassed before use. The mobile phase is supplied by a Shimadzu SCL-10A pump ('s-Hertogenbosch, The Netherlands) at a flow rate of 100 μL / min. (Fig. 2, pump 2). A Waters Associates U6K injector (Etten-Leur, The Netherlands) is placed before the microcolumn (Fig. 2, injector 2) for elution, while an additional Waters U6K injector (Etten-Leur, The Netherlands) (Fig. 2, injector 3) is placed between pump 2 and valve 2 for cleaning the system. Elution of the enkephalins is performed at ambient temperature using a 20 μL solvent plug containing 80% v/v acetonitrile in water. The detection is done with an Applied Biosystems UV detector Model 757 (Separations, H.I. Ambacht, The Netherlands), set at a wavelength of 215 nm (Fig. 2, detector 2).

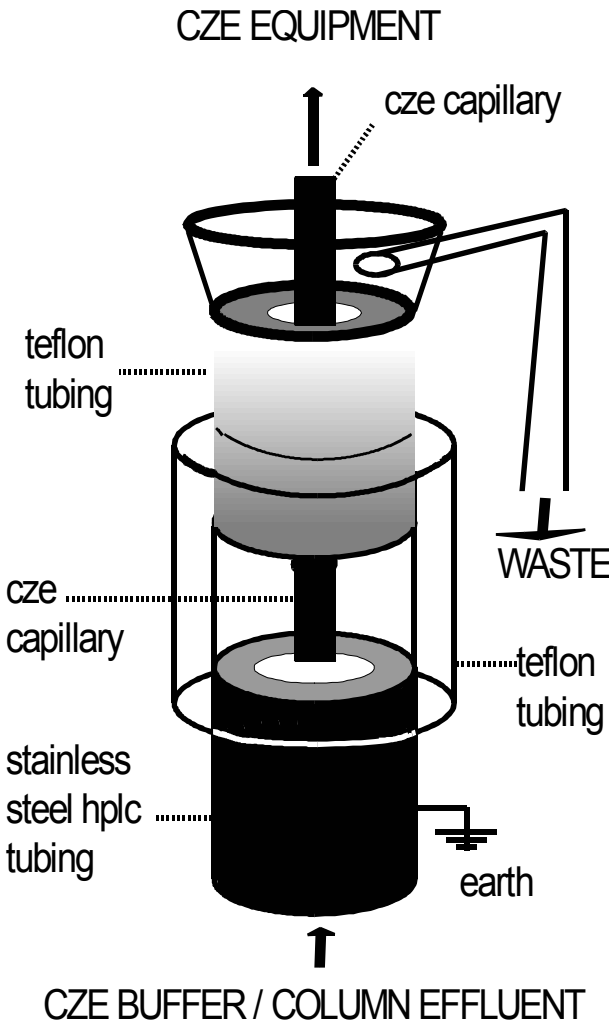

Fig. 3. Schematic representation of the LC-CZE interface

2.2.3 Capillary zone electrophoretic interface

The home-made CZE interface (Fig. 3) is connected to valve 2 (Fig. 2) with stainless steel tubing (0.13 mm i.d., 1.59 mm (1/16") o.d.). The tubing supplies the effluent from the micro column or the CZE buffer. This buffer (solvent C) contains 150 mM phosphoric acid and is adjusted with 2 M NaOH to pH 3.1. Solvent C is supplied by a Waters Associates Model M6000 pump (Etten-Leur, The Netherlands) (Fig. 2, pump 3) at a speed of 1000 μL/min. Simultaneously, the tubing serves as a grounded electrode. The interface is constructed of 20 mm of teflon (PTFE) tubing (1.52 mm (0.060") i.d., 3.18 mm (1/8") o.d.) At the bottom of this piece of PTFE tubing the stainless steel tubing is inserted for 4 mm and at the top a piece of PTFE tubing (20 mm, 0.38 mm (0.015") i.d., 1.59 mm (1/16") o.d.) is inserted for 10 mm, resulting in an interface compartment with a volume of 11 μL. On top of the interface a

device is placed to collect the sample excess and put it to the waste (Fig. 3). The CZE capillary is inserted in the PTFE tubing to a distance of 2 mm from the steel tubing, as indicated in Fig. 3.

2.2.4 Capillary zone electrophoretic system

A PrinCE capillary electrophoretic system (PrinCE Technologies, Emmen, The Netherlands) is used. The capillaries are cut from a batch of fused silica capillaries (50 µm I.D., 375 µm O.D.), purchased from Bester B.V. (Amstelveen, The Netherlands) A total length of 90 cm is employed and a detector window is made at 45 cm from the inlet side of the capillary. The one lift mode of the PrinCE system is used. A potential of -30 kV is applied to the outlet side of the capillary, while the inlet side is grounded. Detection is performed with an Applied Biosystems 785 A Absorbance Detector (Separations, H.I. Ambacht, The Netherlands), adjusted for use in CZE and set at a wavelength of 215 nm.

2.3 Procedures

In the first position of Fig. 2, a CSF sample containing the model peptides is injected (t_0). Subsequently, separation between the peptides and the large endogenous proteins is performed and the protein fraction is transported to the waste. At 2.25 min after t_0, valve 1 is switched to the second position. The effluent containing the peptides is transported by pump 1 through injector 2 to the RP18 trapping column. In the mean time pump 3 transports the CZE buffer (solvent C) through the CZE interface. At 3.25 min after t_0 valve 1 is switched again to the first position and pump 2 purges solvent B through the trapping column *via* detector 2 to the waste to remove the ammonium acetate. At 9.50 min after t_0 a 20 µL plug of 80% acetonitrile in water is injected *via* injector 2 and the effluent from the RP18 column is monitored by detector 2 . Valve 2 is switched at 10.50 min after t_0, while the voltage (-30 kV) of the CZE apparatus is applied with a ramp of 3 kV/s. At this point the third position is achieved. Subsequently, solvent B, containing the elution plug, is transported to the CZE interface, where electrokinetic injection of the peptides from the interface into the CZE is performed. Subsequently, valve 2 is switched at 11.25 min after t_0, thus re-establishing the first position of the system, after which the CZE separation of the enkephalins is completed. Finally, the trapping column is cleaned by injection of a 300 µL plug of 80% acetonitrile in water *via* injector 3.

2.4 Sample pretreatment

Validation of the procedure was done with CSF samples containing the six enkephalins in various amounts, while imipramine HCl was added up to a concentration of 0.02 M. The samples were stored at -18^{o} C and analyzed at ambient temperature.

3. Results and discussion

3.1 System maintenance

In general, the performance of the SEC-CZE system for the analysis of enkephalins in CSF is quite satisfactory. However, after 20-30 sample injections the pressure of both the SEC and the trapping system tends to increase. A thorough flush of the injector and the LC tubing with a sodium dodecyl sulfate (SDS) solution (1% w/w) was found sufficient to clean these systems. Pollution of the capillary wall during injection from the interface also occurs due to endogenous compounds. This results in a slight rise of the migration times due to drop of the electroosmotic flow. Moreover, since the electrokinetic injection from this interface consists mainly of an electroosmotic component, the injection yield from the CZE interface is decreased. This can be prevented by an additional cleaning of the trapping column after each run. This cleaning is performed by injection of a 300 µL plug of 80% acetonitrile in water *via* injector 3 after each run.

3.2 Sample stability

According to the literature the degradation of enkephalins is accelerated by various types and subtypes of peptidases [27-29]. It is impossible to inhibit all enzymes completely. However, a complete inhibition is not necessary. It is acceptable to work with a degradation rate that can be neglected during the course of the analysis in view of the precision of the analytical method. Similar to [25], a protective concentration of 0.02 M imipramine HCl was chosen. At this concentration the enkephalin degradation was negligible during the course of the analysis.

3.3 Application of the separation system

Fig. 4 shows the SEC separation after injecting 10 µL of a CSF sample containing 10 µg/mL of each enkephalin and 0.02 M imipramine HCl. In accordance with the previously reported results [25] peak A represents large proteins such as albumin, peak B represents small peptides such as the enkephalins and peak C consists of imipramine.

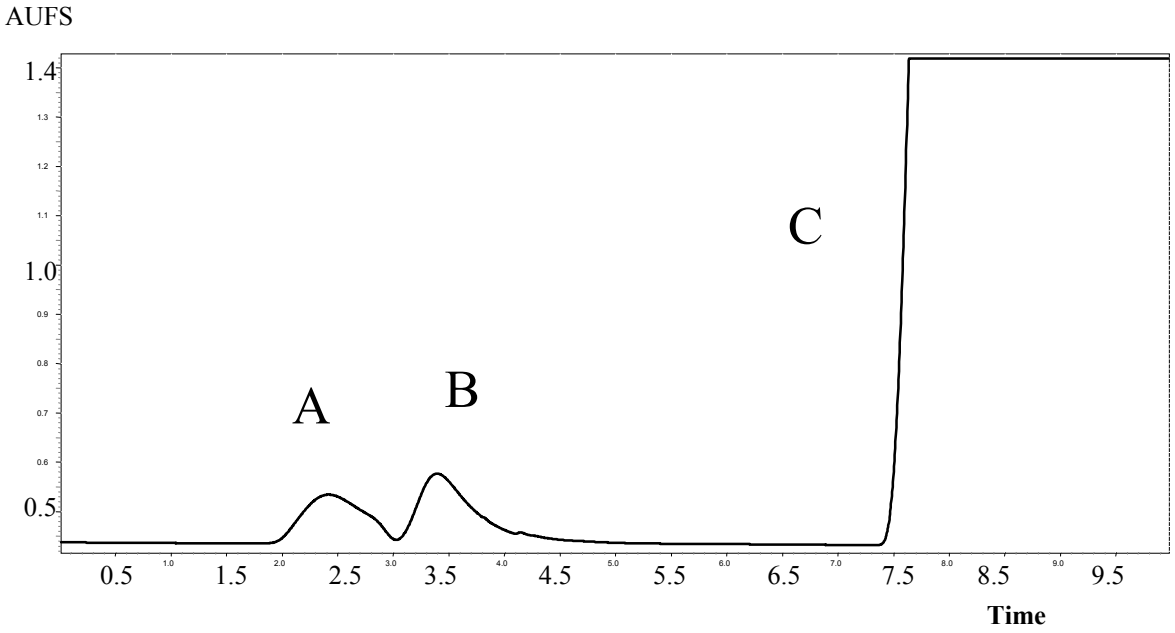

Fig. 4. Chromatogram of the SEC-separation. A: large proteins. B: small peptides (enkephalins). C: imipramine

The separation between the enkephalins in the total system after injection of 10 µL of a CSF sample containing 5 µg/mL of each enkephalin is given in Fig. 5. There is no significant difference with the previously published electropherogram of the separation of a standard solution containing the model enkephalins and albumin [26], implicating the efficient sample clean-up by the SEC dimension, including the removal of the large amount of imipramine HCL. Although endogenous CSF components influence the CZE migration times of the enkephalin peaks [26], these migration times are sufficiently constant to allow quantitation of the compounds (Table II).

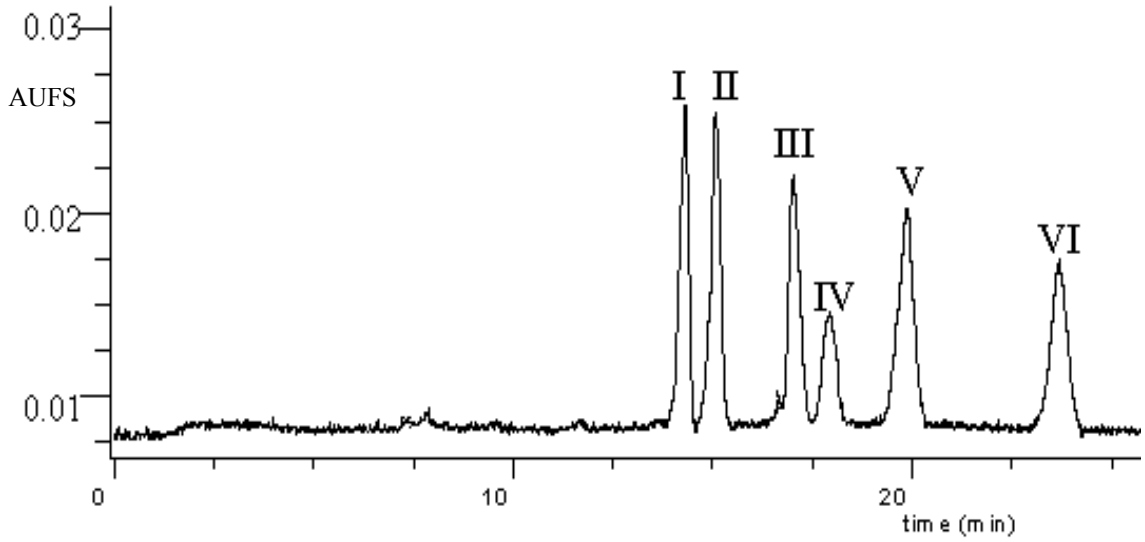

Fig.5. Electropherogram of six enkephalins after injection of 10 µL of a CSF sample spiked with 5 µg/mL of each enkephalin, using the complete system as described in the Materials and Methods section. I: des-Tyr[D-Ala^2-D-Leu^5]-enkephalin, II: des-Tyr-Met-enkephalin, III: [Ala^2]-Leu-enkephalin, IV: Leu-enkephalin, V: [Ala^2]-Met-enkephalin, VI: Met-enkephalin

3.4 Validation of the procedure

The procedure was validated for its linearity and intra- and interday precision. *Linearity* was determined over about one and a half decade (2.5-50 µg/mL). The results are presented in Table I. In general, the correlation is satisfactory. *Precision* of the system was determined intraday and interday by use of peak heights. This was performed with CSF samples containing 10 µg/mL of each enkephalin, while imipramine HCl was added up to a concentration of 0.02 M. The results are presented in Table II. As expected, the average precision of the intraday assay is better than the interday precision. For the determination of the enkephalins in CSF during several days and using one calibration curve, the RSD for the interday precision is relatively high. Consequently, it is necessary to construct a calibration curve per day to obtain reliable results. The decreased precision of the analysis of enkephalins in CSF in contrast to the results for standard solutions presented earlier with this system is supposedly due to the principle of electroosmotic CZE injection applied in this system [26]. Small differences in electroosmotic flow caused by varying adsorption of compounds at the capillary wall will influence the injected amount of enkephalins, while the influence on the RSD of the migration times is low due to a very low EOF in comparison

with the electrophoretic mobility. The problem of irreproducible injection can not be overcome in an easy way. One way might be the replacement of the elektrokinetic injection method for a hydrodynamic one but the latter is virtually impossible in this system. Another way might be a more selective trapping and elution of enkephalins at the trapping device thus preventing the capillary from being polluted by endogenous CSF compounds.

The limit of detection (LOD) is 15 ng and is similar for all used enkephalins. This value was established, using a signal-to-noise ratio (S/N) of 3. The limit of quantitation (LOQ) turned out to be 25 ng (s/n of 5), resulting in a concentration LOQ (CLOQ) of 2.5 μg/mL. In principle, the injection of larger samples into the SEC dimension, improves the LOQ of the enkephalin analysis, but after injecting amounts exceeding 10 μL, serious pollution of the capillary occurred, resulting in irreproducible migration times preventing reliable quantitation of the enkephalins. Consequently, the LOQ cannot be improved by increasing the injected sample volume.

Table I
Linearity: equation, standard deviations and correlation. Y, a, Sd, X and b represent respectively peak height, slope of the line, standard deviation, concentration (μg/mL) of the enkephalin and intercept

2.5 –50 μg/mL, n=12	Y = (a ± Sd a) X + b ± Sd b	R^2
Met-enkephalin	Y = (84.2 ± 2.6) X + 0.18 ± 0.63	0.99
Leu-enkephalin	Y = (77 ± 5.0) X + 0.28 ± 0.14	0.98
des-Tyr[D-Ala^2-D-Leu^5]-enkephalin	Y = (58.7 ± 4.6) X + 0.54 ± 11	0.94
[Ala^2]-Leu-enkephalin	Y = (57.1 ± 2.5) X + 0.25 ± 0.60	0.98
[Ala^2]-Met-enkephalin	Y = (68.6 ± 3.8) X + 0.51 ± 0.93	0.97
des-Tyr-Met-enkephalin	Y = (107.1 ± 5.5) X + 0.13 ± 0.11	0.97

Table II
Precision of the system for peak areas and migration times

	Intraday 10 μg/mL RSD (%, n = 5)	Interday 10 μg/mL RSD (%, n = 5)	Migration-time RSD (%, n = 5)
Met-enkephalin	13	26	1,6
Leu-enkephalin	6.8	21	1.9
des-Tyr[D-Ala^2-D-Leu^5]-enkephalin	9.7	27	2.3
[Ala^2]-Leu-enkephalin	12	24	1.2
[Ala^2]-Met-enkephalin	15	27	2.1
des-Tyr-Met-enkephalin	13	28	2.3

4. Conclusion

The on-line coupled multi-dimensional separation system, presented in this study, is suitable for the quantitation of various enkephalins in CSF with a satisfactory linearity and intraday precision. The results show that it is possible to analyze these closely structural related peptides directly in biological matrices such as CSF, indicating the large selectivity of this rapid multi-dimensional system. The CLOQ of the enkephalins in this system is 2.5 μg/mL, which is comparable to the CLOQ in the earlier presented SEC-LC system [25] and sufficient for the analysis of exogenous peptides used as drugs [30-32]. Both CLOQ values cannot be increased by increasing the injection volume higher than 10 μL. Although the SEC-CZE system contains an additional C18 trapping dimension, offering possibilities for concentration and addition of selectivity in comparison with the loop applied in the SEC-LC system [25], the SEC-CZE system shows the same overall sensitivity due to the inefficient trapping procedure. However, in view of the difficulties caused by the large differences in system properties between CZE and LC systems in general, the results with the more orthogonal and efficient SEC-CZE system in contrast to the SEC-LC system are promising. The CLOQ may be improved by using more selective and sensitive detection methods such as mass spectrometry.

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Conclusions and future perspectives

The objective of this thesis has been the development of novel on-line multidimensional systems for the separation and quantitation of peptides in biological samples. The systems required for quantitation of target peptides generally have different characteristics compared to those which are suitable for mapping of peptides. For mapping a semi-quantitative detection is often sufficient, while comprehensiveness of the system is very important. For multidimensional separation with target quantitation of a certain peptide it is necessary to have a validated quantitative method, while heart-cutting is often sufficient.

Two on-line two-dimensional (2D) systems have been developed: a size exclusion chromatography (SEC)-reversed phase liquid chromatography (RPLC) system and a SEC-capillary zone electrophoresis (CZE) system. In both cases the heart-cut principle is used to isolate in the SEC dimension the fraction, containing six enkephalin peptides as target compounds, from proteins and other interfering substances. This fraction is transferred to the second (RPLC or CZE) dimension and its components are separated. In the SEC-RPLC system a loop is used to transfer the enkephalin-containing fraction, while the SEC-CZE system is based on a new type of interface consisting of trapping of the enkephalins on a micro column and on-line electrokinetic injection of a part of the eluate from this column. Determination of model enkephalins, developed with both systems show satisfactory linearity and intraday precision while the total analysis time is less than 30 min. The concentration limit of detection (CLOD) of both systems, employing UV detection, is in the order of 2-3 μg/mL, which is comparable to other peptide assays based on separation and suitable for the determination and quantitation of a number of peptide drugs with plasma concentrations in the low μg/mL range [1-3].

Both methods allow the injection of CSF samples without pretreatment. The SEC-RPLC system is fully automated, the SEC-CZE system can be automated as well. Direct injection of plasma samples has not been reported, however it may also be possible. However, due to the limited sensitivity of the methods the concentrations of endogenous enkephalins in plasma and CSF cannot be determined, since these concentrations are at least three orders of magnitude lower than the presented CLOD values [4]. It is therefore necessary to improve the sensitivity of the systems. This can be done either by increasing the amount of introduced sample and/or by increasing the detection sensitivity. Due to interferences of endogenous components the sample amount cannot be increased. Ultrafiltration or the use of Restricted

Access Materials (RAM) to exclude (endogenous) proteins and to concentrate the peptides could be options to further minimize these interferences. The most promising approach may be the application of a selective on-line preconcentration step, e.g. affinity chromatography. The introduction of a more sensitive detection mode, such as MS or fluorescence, is the other approach to improve the total sensitivity of the system. Since peptides often are not fluorescent, derivatization of the compounds is necessary. This can be done either pre- or on-column or after the separation (post-column).

An effort was made to increase the sensitivity of the SEC-RPLC system using post- column derivatization with o-phthaldialdehyde (OPA). Pre-column or on-column derivatization might be possible as well, however using these types of derivatizations the RPLC dimension has again to be optimized for the derivatives. In general, the disadvantages of post-column labeling are the negative effect on peak width, incomplete reactions due to short reaction times and the higher baseline signal caused by the added reagent solution. Although OPA is considered to be fluorogenic, its presence increases the background signal by a factor of three [5]. Due to this increased background signal and the peak broadening as a result of the addition of reagent to the LC effluent, a larger sensitivity could still not be achieved for the enkephalins. Further optimization and the use of LIF detection instead of conventional fluorescence detection may give an improvement [6]. The use of a mass spectrometer as detector seems more promising and the mobile phase of the RPLC system contains only volatile salts.

Since the use of MS detection may increase the concentration sensitivity and contributes to the overall selectivity of the separation system, the possibilities for on-line coupling of a MS detector with the SEC-CZE system were investigated. However, the main problem in the development of an on-line coupled LC-CZE-MS system is the different voltage requirements of the CZE and the MS equipment. Both the LC system and the MS have to be electrically grounded, while for electrophoretic separation it is necessary to apply a high voltage at one of the ends of the CZE capillary. This problem might be overcome by using a micro valve with electrically insulated channels at one of the ends of the capillary which enables the electrically separated coupling of the CZE and the MS. The applicability of such a valve is successfully demonstrated for direct in-line injection prior to CZE separation [7]. The internal fluid-contacting materials in this valve are composed of ceramics and polyetheretherketone (PEEK), that are expected to electrically insulate the channels. In general, there are two possibilities to develop a LC-CZE-MS system with the aid of the micro valve: (i) The valve can be placed between the LC dimension and the CZE dimension. This set-up has the

advantage that the negative effect of any peak broadening due to the passage of analytes through the valve on the sensitivity can be reversed by stacking in the CZE dimension. (ii) Another, however less favorable, set-up is positioning of the valve between the CZE capillary and the MS. In this case minimization of peak broadening due to passage of the analytes through the valve is crucial because stacking can not decrease this peak broadening. Some system set-ups in which the positioning of the valve in the set-up was varied, were briefly investigated. The preliminary conclusion is that the micro valve, placed at the beginning of the CZE separation capillary, may be useful as an in-line injector in a coupled LC-CZE-MS system when low voltages (<8kV at the valve) and separation buffers with both a relatively high pH and a low concentration are employed. However, from this study it appeared that with high currents and, even more, at low electroosmotic flow, there is current leakage from the valve to earth. There is also a considerable peak broadening during passage of the analytes through the valve. Therefore, positioning of a micro valve at the end of the capillary to enable the injection of a sample from the CZE effluent into the MS is not advisable.

The present separation systems are feasible for the quantitation of a specific set of peptides. If other peptides have to be quantified, the systems require adjustments. Comparison of the SEC-RPLC system with the SEC-CZE system makes clear that the RPLC dimension in the SEC-RPLC system can be adjusted by changing the nature of the column, the amount of modifier in the mobile phase and its pH. The CZE dimension in the SEC-CZE dimension is less flexible to adjustments, because of the presence of the trapping column. The pH of the CZE buffer should have the same value as the pH of the mobile phase for the trapping column and the preconcentration of the enkephalins was optimized for this trapping column under these conditions. In general, the power of the systems can be further increased by the use of MS detection. Using the SEC-RPLC system this coupling with MS seems easy to accomplish. For the SEC-CZE system this may be realized by the use of the in-line valve and a suitable run buffer.

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Samenvatting

Dit proefschrift beschrijft de ontwikkeling en de toepassing van on-line gekoppelde multidimensionale scheidingssystemen voor de analyse van proteïnen en peptiden. Het doel van het onderzoek was de ontwikkeling van bruikbare on-line gekoppelde multidimensionale scheidingssystemen voor de analyse van peptiden. De multidimensionale scheidingssystemen zijn van belang voor het vakgebied proteomics. Het vakgebied proteomics richt zich op onderzoek naar de veranderingen in eiwitexpressies die o.a. ontstaan bij bepaalde ziekten. Bij proteomics heeft men te maken met het voorkomen van complexe analytische problemen. Het is namelijk niet eenvoudig om de, vaak geringe kwantitatieve en/of kwalitatieve, veranderingen in eiwitexpressie te detecteren. De aanwezigheid van een zeer gevoelig en selectief analytisch systeem is, in dit geval, noodzakelijk. Een andere belangrijke reden voor de ontwikkeling van deze systemen is de toepassing als analytisch systeem voor peptide of proteïne geneesmiddelen. Voor deze groeiende groep geneesmiddelen is o.a. farmacokinetisch en biofarmaceutisch onderzoek voorafgaand aan het op de markt komen van een nieuw geneesmiddel noodzakelijk. Analyse van deze peptiden of eiwitten is ook van belang bij therapeutic drug monitoring. Selectieve, gevoelige en snelle analyse methoden die gebaseerd zijn op de on-line gekoppelde multidimensionale scheidingssystemen zijn hiervoor vaak noodzakelijk.

Scheidingssystemen kunnen volgens verschillende benaderingen on-line met elkaar gekoppeld worden. **Hoofdstuk 1** geeft een overzicht de meest gangbare benaderingen. De eerste sectie beschrijft de systemen bestaande uit een hoge-druk vloeistofchromatografisch systeem gekoppeld aan eveneens een hoge-druk vloeistofchromatografisch systeem. De tweede sectie beschrijft de hoge druk vloeistof chromatografie-capillaire zone elektroforetische systemen en de derde sectie geeft een overzicht van de koppelingen tussen capillaire zone elektroforetische systemen onderling. In hoofdstuk 1 ligt de nadruk bij de ontwikkeling van de systemen, terwijl de toepasbaarheid ook een belangrijke rol krijgt. Omdat voor een gevoelige detectie een massaspectrometer (MS) vaak noodzakelijk is, is de combinatie van MS met de beschreven systemen ook beschreven. Het blijkt dat de systemen met capillaire zone elektroforese steeds belangrijker worden, alhoewel de systemen die bestaan uit louter vloeistofchromatografie eenvoudiger te realiseren zijn en bovendien gemakkelijker te koppelen aan een MS. De keuze voor een bepaald gekoppeld systeem is

complex en hangt voornamelijk af van de begeleidende stoffen die aanwezig zijn in het monster en het doel van de analyse.

Capillaire zone elektroforese is een techniek die veel wordt toegepast binnen de gekoppelde systemen. Dit komt voornamelijk door de grote scheidingsefficiëntie. De detectielimiet in concentratie-eenheden is echter relatief hoog. Door preconcentrering kan de concentratiegevoeligheid in het algemeen verbeterd worden. **Hoofdstuk 2** geeft daarom een overzicht van mogelijke on-line en in-line preconcentreringstechnieken die worden toegepast bij capillaire zone elektroforetische systemen. Deze preconcentreringstechnieken zijn te verdelen in twee groepen. De preconcentrering kan namelijk berusten op het chromatografische principe of het elektroforetische principe. Ook hier hangt de keuze van een bepaalde preconcentreringstechniek af van de samenstelling van het monster. Indien het monster bijvoorbeeld veel zouten bevat, heeft een chromatografische preconcentrering de voorkeur.

Om een bijdrage te leveren aan de vooruitgang op het gebied van de on-line multidimensionale scheidingssystemen zijn er tijdens dit onderzoek twee nieuwe systemen ontwikkeld. **Hoofdstuk 3** beschrijft de ontwikkeling en optimalisering van een heart-cut multidimensionaal gekoppeld systeem dat bestaat uit een size-exclusion chromatografisch systeem gevolgd door een reversed-phase chromatografisch systeem. De interface bestaat uit twee loops met twee zeswegkranen. Zes verschillende peptiden (enkephalines), die qua structuur weinig van elkaar verschillen, kunnen met dit systeem goed worden gekwantificeerd in een waterig monster waaraan albumine is toegevoegd. Het eerste systeem scheidt de enkephalines van de albumine, terwijl het tweede systeem de enkephalines onderling scheidt. Dit systeem is volledig geautomatiseerd. In **hoofdstuk 4** wordt de toepassing van dit systeem beschreven voor de analyse van de peptiden in cerebro-spinaal vocht. De minimale concentraties van de enkephalines die met dit systeem in cerebro-spinaal vocht kunnen worden gekwantificeerd is 1 μg/mL. De cerebro-spinaal vocht monsters kunnen direct worden geïnjecteerd. Eventueel is directe injectie van plasma monsters mogelijk.

Hoofdstuk 5 beschrijft de ontwikkeling en optimalisering van een tweede heart-cut multidimensionaal gekoppeld systeem dat bestaat uit eveneens een size-exclusion chromatografisch systeem, echter nu gevolgd door een preconcentrering, met reversed-phase chromatografie (micro-kolom), en een capillaire zone elektroforese voor de scheiding van de peptiden. Om de koppeling tussen het chromatografische en het elektroforetische systeem mogelijk te maken is er een nieuwe interface ontwikkeld en geoptimaliseerd. Het eerste

systeem scheidt de enkephalines weer van de albumine, vervolgens worden de enkephalines tegelijk geconcentreerd op de reversed-phase kolom terwijl het elektroforetische systeem de enkephalines onderling scheidt. Dit systeem is niet geautomatiseerd, echter het is wel mogelijk. **Hoofdstuk 6** beschrijft de toepassing van dit systeem voor de analyse van enkephalines in cerebro-spinaal vocht. De minimale concentraties van de enkephalines die met dit systeem in cerebro-spinaal vocht kunnen worden gekwantificeerd is 2,5 μg/mL. De cerebro-spinaal vocht monsters kunnen ook in dit systeem direct worden geïnjecteerd.

De beide nieuwe systemen zijn goed bruikbaar, echter de gevoeligheid voor de kwantificering van endogene enkephalines in cerebro-spinaal vocht is (nog) niet voldoende. De **Conclusies en toekomstige perspectieven (Conclusions and future perspectives)** geven aanbevelingen m.b.t. de verbetering van de gevoeligheid van beide systemen. Sommige aanbevelingen zijn nader uitgewerkt: als mogelijke verbetering voor het eerste systeem wordt de toevoeging van een on-line pre-kolom derivatiseringsreactie uitgezocht en als mogelijke verbetering van het elektroforetische systeem is de on-line koppeling met een massaspectrometer uitgezocht.

Curriculum Vitae

Thom Stroink werd geboren op 31 juli 1971 te Zutphen. Na het doorlopen van het VWO aan de Koninklijke Scholengemeenschap Apeldoorn werd in 1991 het diploma behaald. In datzelfde jaar is er begonnen met de farmaciestudie te Utrecht. Het propedeutisch examen werd binnen een jaar behaald. Het vierde jaar van de farmaciestudie te Utrecht bestond uit een zes maanden durend onderzoeksproject, twee keuzepakketten en het schrijven van een scriptie.

Het onderzoeksproject is uitgevoerd bij het Research Centre for Natural Products and Phytopharmaceuticals van de Universiteit Utrecht met als onderwerp het optimaliseren van de isolatie van de actieve stof filipentoline uit de wortels en bladeren van *Filipendula ulmaria (*moerasspirea). Daarnaast is een chromatografisch systeem ontwikkeld voor de kwantificering van filipentoline in de bladeren en wortels van deze plant. De twee keuzepakketten werden gelopen bij de Stability Research Group te Utrecht. Bij deze groep is onderzoek gedaan naar de ontledingssnelheid van acetylsalicylzuur in aanwezigheid van verschillende cyclodextrinen en naar de chemische stabiliteit van adrenaline in oogdruppels. De scriptie geeft een beschouwing van de mogelijke werkingsmechanisme van de hoge potenties in de homeopathie.

In april 1996 is Thom met genoegen geslaagd voor het doctoraal examen farmacie. Tijdens de studie is hij verschillende keren student-assistent geweest.

Na het doctoraal examen is Thom een half jaar werkzaam geweest als visiting scientist aan de universiteit van Kumamoto (Faculty of Pharm. Sciences) te Japan. Er is hier een analytische methode ontwikkeld om de biologische beschikbaarheid van een nieuwe ontstekingsremmer (KCA-098) uit het maagdarmkanaal te kunnen bepalen.

Na dit bezoek is hij verder gegaan met de tweede fase opleiding tot Apotheker. Verder heeft Thom in deze periode patientenbijsluiters geschreven voor de stichting Health Base. Het apothekersdiploma werd behaald in april 1998. Na het behalen van het apothekersdiploma is Thom kortdurend werkzaam geweest als junior docent bij het postdoctorale recepteerkunde onderwijs. In de herfst van 1998 werd begonnen bij de disciplinegroep Biomedische Analyse, een onderdeel van de Faculteit Farmaceutische Wetenschappen te Utrecht, met het onderzoek dat geleid heeft tot de verschijning van dit proefschrift.

Momenteel werkt Thom als docent bij de disciplinegroep Biofarmacie en Farmaceutische Technologie van de Faculteit Farmaceutische wetenschappen te Utrecht.

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