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Journal of Chromatography B, 839 (2006) 30–35
JOURNAL OF CHROMATOGRAPHY B
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On-line coupling of size exclusion chromatography and capillary electrophoresis via solid-phase extraction and a Tee-split interface☆

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Received 14 November 2005; accepted 2 February 2006
Available online 2 March 2006

Abstract

An on-line size exclusion chromatography (SEC)–solid-phase extraction (SPE)–capillary electrophoresis (CE) system using a Tee-split interface has been developed for the analysis of peptides in biological fluids. The SEC column fractionates the sample by molecular size and the low-molecular-weight fraction, which contains the peptides, is directed to a C_{18} SPE microcolumn, where the peptides are trapped and concentrated. The SPE column is desorbed with 425 nL acetonitrile and the effluent is sent to the Tee-split interface, which hydrodynamically splits (1:40) the flow and, thus, allows appropriate injection of analytes into the CE system. The performance of the system is investigated by the analysis of enkephalins in cerebrospinal fluid (CSF). It is demonstrated that the SEC step efficiently removes potentially interfering proteins, permitting reproducible SPE and CE. The total system provides efficient separations of the enkephalins with plate numbers up to 100,000. Concentration limits of detection (S/N = 3) for the peptides are about 100 ng/mL for injection of 20 μL spiked CSF samples. Plots of enkephalin peak areas versus concentration showed good linearity over the 0.25–10 μg/mL range ($R^2 \geq 0.985$). Repeatability of migration time and peak area was within 2% and 10% R.S.D., respectively.

Keywords: Size exclusion chromatography; Solid-phase extraction; Capillary electrophoresis; Tee-split interface; Enkephalin; Cerebrospinal fluid

1. Introduction

Capillary electrophoresis (CE) is an adequate alternative for established separation techniques such as liquid chromatography (LC) because of its high separation efficiency, short analysis time and low sample and buffer consumption. A major drawback of CE is the low concentration sensitivity, which is mainly caused by the limited sample volume that can be introduced into the capillary and, when UV absorbance detection is used, the short optical path length. The sensitivity can be improved by longer optical path lengths for UV detection, more sensitive detection methods (e.g. laser-induced fluorescence or mass spectrometry), or analyte preconcentration.

Sample concentration can be effectively achieved by means of a small solid-phase extraction (SPE) column. Relatively large sample volumes can be loaded on such columns, where the analytes are retained and accompanying compounds, e.g. salts, removed. Upon desorption of the analytes from the column with an eluting solvent, the analytes can be concentrated in a small volume plug, leading to low detection limits. For the combination of SPE and CE, in-line [1–4] and on-line [5–12] couplings are preferable because of automation possibilities and a minimum of sample handling. In-line SPE–CE systems, in which the SPE column is incorporated in the CE capillary, have the major drawback that the sample matrix has to be introduced into the separation capillary that may adversely influence the CE separation efficiency and reproducibility. Recently, we have coupled SPE and CE in an on-line fashion using either an in-line valve [11] or a Tee-split interface [12]. Using these on-line systems, a sample volume of 250 μL could be concentrated to approximately 1 μL, which then was partially introduced into the CE system. So far, the principles and potential of these

☆ This paper was presented at the 4th International Symposium on Separations in the BioSciences (SBS '05), Utrecht, The Netherlands, 18–21 September 2005.

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doi:10.1016/j.jchromb.2006.02.003

systems have been outlined using aqueous enkephalin samples. When biological samples have to be analyzed, additional sample pretreatment prior to the SPE step may be needed to remove compounds that jeopardize an effective analyte concentration (or even block the SPE column) and the subsequent CE analysis. Sample pretreatment prior to SPE can be achieved by carrying out a preceding separation. Generally, sample analysis with on-line multidimensional separation systems can be performed using a comprehensive or a heart-cut approach. The comprehensive approach results in the analysis of the complete sample in all subsequent dimensions, whereas the heart-cut approach analyzes only a small part of the pre-separated sample in the second separation step. The comprehensive approach demands a slow preceding separation compared to the subsequent separation in order to accomplish analysis of the complete sample in all dimensions. Typical examples of such comprehensive systems are the on-line size exclusion chromatography (SEC)–CE systems and reversed phase LC–CE systems developed in the group of Jorgenson [5,13–19], which are coupled by various interfaces. These systems do not concentrate the chromatographic fractions prior to introduction into the CE system, which reduces the sensitivities of the total systems. Efforts to integrate such a focusing step would imply the need for an even slower preceding separation step to create time for sample trapping in a SPE column, washing and desorption of the concentrated fraction and sample introduction into the CE system. In practice, a comprehensive multidimensional system with a focusing step seems almost impossible, unless a number of columns are integrated into the system in a parallel fashion to enable "parking" of the LC fractions. In that case, the LC fractions are stored in the focusing step on various SPE columns and can be sent to the CE system at any convenient moment.

The heart-cut approach is less demanding and is best suitable for target analysis. In the case of a heart-cut approach for an on-line system, it is also easier to integrate a concentration step between the preceding and the final separation step because there are no time constraints. Stroink et al. [20,21] coupled SEC–SPE with CE through a vial-type interface for the quantitative analysis of enkephalins in cerebrospinal fluid (CSF). The SEC dimension separated the sample in a protein- and a peptide-containing fraction. This resulted in a relatively large volume of the peptide fraction (about 200 μL), requiring a subsequent SPE step prior to CE analysis. Quantification limits of 2.5 μg/mL for CSF samples spiked with enkephalins were obtained.

The objective of the present study was to develop an on-line SEC–SPE–CE system for the analysis of peptides in biological fluids, using the on-line SPE–CE system with a Tee-split interface presented earlier [12]. The small SPE column provided effective sample preconcentration using desorption volumes of 425 nL. The Tee-split interface enabled on-line injection of the concentrated analytes into the CE system without disturbing separation efficiency. These features are also favorable for the on-line SEC–SPE–CE system. System development and evaluation has been performed in a few steps. First, the SEC dimension has been examined, and then the on-line SEC–SPE part has been evaluated concerning the fractionation in the SEC dimension and the SPE performance for the (enkephalin) peptide-containing fraction. Finally, the complete SEC–SPE–CE system has been tested and validated for the analysis of CSF samples.

2. Experimental

2.1. Chemicals

Ammonium acetate, boric acid, acetonitrile and sodium hydroxide (all analytical grade or higher) were purchased from Merck (Darmstadt, Germany), OPG Pharma (Utrecht, The Netherlands), Biosolve BV (Valkenswaard, The Netherlands) and Bufa BV (Uitgeest, The Netherlands), respectively. Acetic acid (analytical grade), bovine serum albumin (purity 99%, essential fatty acid and globulin free), and acetate salts of [Met^5]-enkephalin, des-Tyr^1-[D-Ala^2-D-Leu^5]-enkephalin and des-Tyr^1-[Met^5]-enkephalin (purity 99% for all peptides) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Human CSF was generously donated by the Academic Hospital Maastricht (Maastricht, The Netherlands). Deionized and filtered water from a Milli-Q system (Millipore, Bedford, MA, USA) was used for all solutions, unless stated otherwise.

Solvent A was a 16 mM ammonium acetate buffer, which was adjusted to pH 5.8 with acetic acid. Solvent B consisted of an 8 mM ammonium acetate buffer, adjusted to pH 4.8 with acetic acid, to which 15% (w/w) acetonitrile was added. For the CE system 50 mM boric acid buffer, adjusted to pH 8.0 with sodium hydroxide, was used as background electrolyte (BGE).

2.2. Apparatus

The on-line SEC–SPE–CE system (Fig. 1) was built in three distinct parts: a SEC, a SPE and a CE part. The SEC part consisted of a pump (model LC-10ADvp; Shimadzu, Kyoto, Japan) (pump 1), a valve (model 7010; Rheodyne, Rhonert Park, CA, USA) (valve 1) for introduction of sample, a SEC column (type BioSep SEC-S2000, 30 mm × 4.6 mm i.d., 5 μm particles with

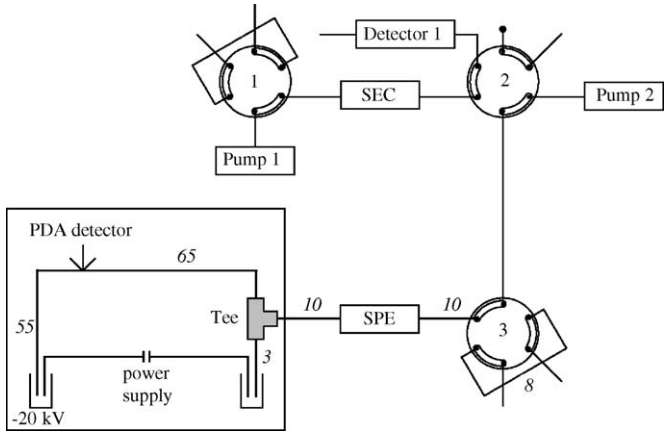

Fig. 1. Schematic diagram of the on-line SEC–SPE–CE system with the Tee-split interface. The CE part of the complete system is framed. Lengths of fused silica capillaries (75 μm i.d.) are shown in italics (cm). All other capillaries are made of PEEK (125 μm i.d.). System components such as valves, detectors, columns, the Tee-split interface and CE apparatus are described in Section 2.

145 Å pores; Phenomenex, Torrance, CA, USA), and a UV detector (model 759A; Applied Biosystems, Foster City, CA, USA) (detector 1) set at a wavelength of 215 nm. The SPE part comprised a pump (model LC-10ADvp; Shimadzu) (pump 2), a micro valve (model Cheminert C2-0006; VICI, Houston, TX, USA) (valve 3) for introduction of acetonitrile, and a SPE column (type PepMap C_{18}, 5 mm × 0.5 mm i.d., 5 μm particles with 100 Å pores; LC Packings-A Dionex Company, Amsterdam, The Netherlands). Valve 2 (model 7010; Rheodyne) functioned as a selection valve to direct a fraction of solvent A towards the SPE column or to detector 1. The CE part consisted of a CE system (model G1600Ax; Agilent Technologies, Waldbronn, Germany) with a build-in photodiode array detector set at a wavelength of 200 nm. A homemade assembly of a 15 mL glass vial and an electrode served as the grounded inlet of the CE system, which was positioned outside the apparatus. A dedicated cartridge was used to guide the separation capillary out of the CE apparatus. The CE and SPE parts were connected by a polyetheretherketone (PEEK) micro Tee with a void volume of 29 nL (model P-775; Upchurch Scientific Inc., Oak Harbor, WA, USA). In this system, the micro valve and the SPE column were grounded as safety precaution. Fused silica capillaries (75 μm i.d., 375 μm o.d.; BGB Analytik, Anwil, Switzerland) were used in this setup as depicted in Fig. 1. All other connections were made with PEEK tubing (125 μm i.d.; Upchurch Scientific Inc.). The SEC part was filled with solvent A, whereas in the SPE and CE parts BGE was used.

The separation on the SEC column was tested using a conventional set up with an injection valve, the column and a UV detector. For the evaluation of the coupling of the SEC column and the small SPE column, an on-line SEC–SPE–LC system was constructed. This system was similar to the on-line SEC–SPE–CE system (Fig. 1), apart from some modifications. The CE was replaced by an isocratic LC system, which was connected to the SPE part via an additional valve (model 7010; Rheodyne), instead of the micro Tee. Pump 2 was connected to this valve, so that the valve enabled introduction of either the eluent from the SPE column or solvent B from pump 2 into the LC column (type LiChrospher C_{18}, 125 mm × 2 mm i.d., 4 μm particles; Phenomenex). An additional detector (model 759A; Applied Biosystems), set at a wavelength of 215 nm, was used for recording of the LC step.

2.3. Procedures

Daily start-up conditioning of the SEC column consists of successive rinsing with water and solvent A at 50 μL/min for 10 min each, while the SPE column is cleaned five times with a plug of acetonitrile. The LC column is conditioned with solvent B at a flow of 50 μL/min, and the CE system with, successively, 0.1 M sodium hydroxide, water and BGE, introduced at the detector (detector 2) side of the system by applying 1500 hPa pressure for 10 min each.

The procedure for the experiments done with the SEC–SPE–LC system is summarized in Table 1. In short, sample is introduced via the loop of valve 1 and separated on the SEC column at a flow rate of 50 μL/min. A part of the SEC effluent is directed to the SPE column by switching valve 2 and the SPE column is washed with solvent A. Then, the flow is reduced to 3 μL/min and the trapped analytes are desorbed from the SPE column with a plug of acetonitrile from the loop on valve 3 (Fig. 1) and introduced into the LC column. The valve between the SPE and LC column (the LC valve) is switched to allow pumping solvent B through the LC column at a flow rate of 50 μL/min and data acquisition is started.

Table 1
Time schedule for operation of the on-line SEC–SPE–LC system, which set up is similar to Fig. 1, apart from the CE system and the Tee-split interface, which are replaced by an LC system and a valve (the LC valve), respectively

Time (min)	Action	Position		
		Valve 1	Valve 2	Valve 3
	Fill loop of valve 1 with sample; pump 1 on 50 μL/min	0	0	0
0	Inject sample	1	0	0
8.3	Guide solvent A to SPE column	1	1	0
16.8	Flow pump 1 to 3 μL/min and fill loop of valve 3 with acetonitrile	1	1	0
18.8	Inject AcN	1	1	1
20.8	Switch LC valve and start LC analysis (50 μL/min, 45 min)	1	1	1

Valves 1–3 are as shown in Fig. 1. Position 0 = position as depicted in Fig. 1, position 1 = position opposite to position 0.

The procedure for the experiments done with the SEC–SPE–CE system is summarized in Table 2. After injection, the sample is separated by the SEC column. Part of the SEC eluent is directed to the SPE column by switching valve 2, and a 1500 hPa pressure is applied at the CE outlet BGE vial. Then, valve 2 is switched back into its original position and the BGE is guided through the SPE column via pump 2 at a flow rate of 50 μL/min. The flow is reduced to 3 μL/min, the analytes are desorbed from the SPE column with a plug of acetonitrile out of the loop of valve 3 and directed to the Tee-split interface. After injection of the desorbed analytes via the Tee-split interface,

Table 2
Time schedule for operation of the on-line SEC–SPE–CE system

Time (min)	Action	Position		
		Valve 1	Valve 2	Valve 3
	Fill loop of valve 1 with sample; pump 1 on 50 μL/min	0	0	0
0	Inject sample	1	0	0
8.3	Guide solvent A to SPE column and apply 1500 hPa at CE outlet	1	1	0
13.8	Guide the BGE to SPE column via pump 2	1	0	0
16.8	Flow pump 2 to 3 μL/min; fill loop of valve 3 with acetonitrile and remove 1500 hPa at CE outlet	1	0	0
18.8	Inject AcN	1	0	1
20.8	Stop pump 2	1	0	1
21.3	Start CE analysis (−20 kV, 20 min)	1	0	1

Valves 1–3 are as shown in Fig. 1. Position 0 = position as depicted in Fig. 1, position 1 = position opposite to position 0.

pump 2 is stopped and CE analysis is performed at −20 kV for 20 min.

3. Results and discussion

3.1. SEC

To test the separation performance of the SEC column, two different samples, i.e. a sample with 20 μg/mL bovine serum albumin and 50 μg/mL des-Tyr^1-[Met^5]-enkephalin in water and a CSF sample spiked with 50 μg/mL des-Tyr^1-[Met^5]-enkephalin, were selected. Separation was performed using solvent A at a flow rate of 50 μL/min. The relatively low flow rate was chosen to ensure acceptable backpressures when the SEC column is coupled to the SPE column. The performance of the SEC column was similar at flow rates of 50 and 200 μL/min. An injection of 10 μL of the sample with bovine serum albumin and the enkephalin peptide resulted in baseline separation of the protein and the peptide. A 10 μL injection of a blank CSF sample resulted in a chromatogram with two bands (Fig. 2a). The first (around 6 min) originates from the proteins present in CSF and the second (around 10 min) from smaller molecules. A CSF sample spiked with 50 μg/mL des-Tyr^1-[Met^5]-enkephalin was also analyzed by SEC (Fig. 2b). Comparing Fig. 2a and b, the position of the enkephalin peak could be determined (indicated by an arrow in Fig. 2b). Increasing the injection volume to 20 μL did not change the separation efficiency, however, with an injection volume of 50 μL the separation of the protein- and peptide-containing zones was not sufficient. Using injections of 10 or 20 μL, it was concluded that the SEC column allowed the removal of proteins from the CSF sample by valve switching of the effluent (see Sections 3.2 and 3.3).

3.2. SEC–SPE

The development of the SEC–SPE–CE system was continued by combining SEC and SPE. A small C_{18} SPE column was chosen for trapping the SEC fraction containing the peptides at a flow of 50 μL/min. SPE breakthrough volumes were determined to be around 750 μL. The desorption efficiency could not be determined by direct UV detection at 215 nm of the SPE eluent because the signal of the acetonitrile interfered with the signal of the analytes. In order to allow a quantitative evaluation of the SEC–SPE system, a conventional LC–UV system was coupled to SEC–SPE through a valve (the LC valve), which also enabled switching between solvents A and B. The LC system provided separation of the desorbed enkephalins from the acetonitrile used for desorption. In this on-line SEC–SPE–LC system, a valve directs the SEC eluent either to waste via a detector or to the SPE column, thus enabling a selected part of the SEC eluent to be trapped on the SPE column.

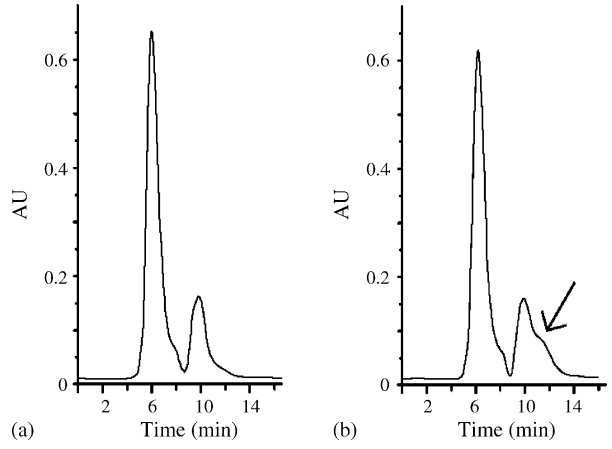

Fig. 2. SEC chromatograms of (a) 10 μL CSF and (b) 10 μL CSF spiked with des-Tyr^1-[Met^5]-enkephalin (50 μg/mL). The arrow indicates the position of the enkephalin peptide. Flow rate, 50 μL/min; detection at 215 nm. For further conditions, see Section 2.

The on-line SEC–SPE step was tested analyzing a mixture of bovine serum albumin (20 μg/mL) and des-Tyr^1-[Met^5]-enkephalin, and a CSF sample spiked with the enkephalin peptide. Both samples yielded LC chromatograms with only one peak (originating from the enkephalin peptide). Apparently, the peptide was transferred to the LC system in absence of bovine serum albumin or CSF components. The enkephalin peptide was also directly injected into the LC column and the enkephalin recovery from the SPE column was determined to be around 65%. This recovery was also found for an SPE–CE system reported earlier [11] and suggests that the enkephalin peptide injected onto the SEC column is trapped quantitatively on the SPE column. The SEC–SPE step showed good linearity (concentration versus peak height) up to 80 μg/mL ($R^2 \geq 0.99$). Repeated analysis yielded an R.S.D. of less than 10% for the peak height of the enkephalin peptide. These results show that the performance of the SEC–SPE part is satisfactory regarding the depletion of proteins by the SEC column and the trapping of the enkephalin peptides by the SPE column.

3.3. SEC–SPE–CE

The on-line coupling of the SEC–SPE part with the CE system is performed using a Tee-split interface (Fig. 1). The interface splits all incoming flow inversely proportional to the ratio of the capillary resistances exiting the Tee. To prevent matrix molecules from entering the separation capillary during SPE trapping and washing, a pressure is applied at the CE outlet vial. When the analytes are desorbed from the SPE column, the pressure at the outlet vial is removed and the flow is splitted in the Tee: the major part flows into the short capillary end to the inlet vial, whereas a minor part runs into the separation capillary for injection. The characteristics of the Tee-split interface have been investigated earlier regarding split ratio and separation efficiency [12]. With a 120 cm capillary from the Tee to the outlet vial and a 3 cm capillary from the Tee to the inlet vial, the split ratio was 1:40. Plate numbers above 100,000 were obtained for enkephalin peptides.

In this SEC–SPE–CE coupling, it is necessary to change eluent after trapping because the eluent from the SPE column is introduced into the CE system via the waste capillary (placed between the inlet vial and the Tee-split interface) during CE analysis. Therefore, a pump is attached to valve 2 (Fig. 1), pumping

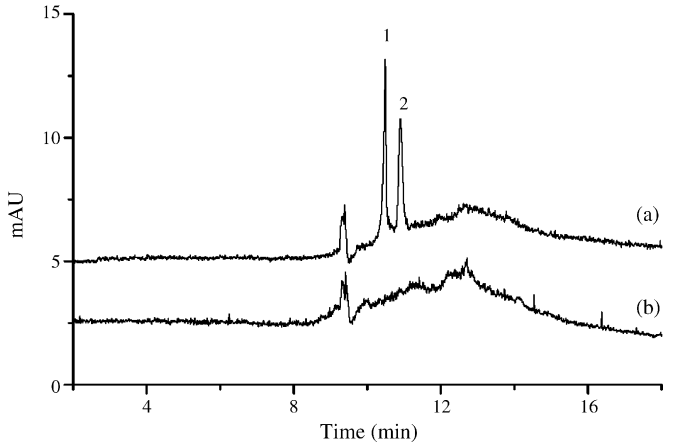

Fig. 3. Electropherogram of (a) CSF spiked with des-Tyr^1-[D-Ala^2-D-Leu^5]-enkephalin (1) and [Met^5]-enkephalin (2), each present at 0.5 μg/mL, and (b) unspiked CSF using the on-line SEC–SPE–CE system. Sample volume, 20 μL; split ratio, 1:40; analysis voltage, −20 kV. For further conditions, see Section 2.

BGE towards the CE system after trapping of the (enkephalin) peptide-containing plug. After SPE desorption and sample injection into the CE system, the analytes that are still in the short capillary end are removed by additional flushing of that capillary via the SPE system. The detailed SEC–SPE–CE procedure is described in the Experimental Section. In short, 20 μL of sample is introduced onto the SEC column. The SEC plug containing the peptides is trapped on the SPE column. Desorption of the enkephalin peptides from the SPE column is performed with 0.4 μL acetonitrile, and the elution plug is partially injected into the CE system via the Tee-split interface. Finally, CE analysis is performed at −20 kV for 20 min.

In Fig. 3a, the separation of a 20 μL CSF sample spiked with two enkephalins, i.e. des-Tyr^1-[D-Ala^2-D-Leu^5]-enkephalin (peak 1) and [Met^5]-enkephalin (peak 2), is shown using the complete on-line SEC–SPE–CE system. The peptides are baseline separated with plate numbers up to 100,000. The electropherogram of an unspiked CSF sample (Fig. 3b) resulted in an electroosmotic flow signal at around 9.5 min and an increased baseline between 10 and 16 min. Comparing Fig. 3a and b, it can be concluded that the enkephalin peptides can be detected without interference of other CSF components. For a 20 μL sample volume and a split ratio of 1:40 in the Tee-split interface, concentration limits of detection (S/N = 3) are around 100 ng/mL. Linearity (area versus concentration) was tested for CSF samples spiked with des-Tyr^1-[D-Ala^2-D-Leu^5]-enkephalin and [Met^5]-enkephalin in the range of 0.25–10 μg/mL, resulting in good correlation. The equations are $y = 71.12x - 13.48$ ($R^2 = 0.985$) and $y = 62.28x + 1.08$ ($R^2 = 0.999$) for des-Tyr^1-[D-Ala^2-D-Leu^5]-enkephalin and [Met^5]-enkephalin, respectively. Repeatability of the on-line SEC–SPE–CE system is examined ($n = 10$) for migration time and peak area, giving values of <2% R.S.D. and <10% R.S.D., respectively. The SEC column has been used for more than 250 injections of CSF. For injections of CSF, the lifetime of the SPE cartridge is increased by the use of the SEC column, because this column prevents that a significant part of the CSF proteins, which may cause blockage, enter the SPE cartridge. In this set up, over 100 injections have been done without an excessive increase in back pressure of the precolumn.

4. Conclusions

An SEC–SPE–CE system is developed for the isolation, concentration and separation of peptides in biological samples. This system results from an extension of a recently developed on-line SPE–CE set up. The SEC column was needed to discard proteins, which are present in biological samples such as CSF. For 20 μL injections of CSF spiked with enkephalins, detection limits were about 100 ng/mL. Separations of peptides show plate numbers of up to 100,000, indicating that the coupling via the Tee-split interface does not cause serious band broadening. Good linearity of the complete on-line SEC–SPE–CE system was demonstrated for CSF sample volumes of 20 μL. Determination of exogenous enkephalins in the low μg/mL range in CSF or plasma is very well possible, but for endogenous enkephalins, which are present in the low ng/mL range, sensitivity improvement is still needed. This may be possible by selecting a more suitable SEC column that can handle larger sample volumes. However, to prevent analyte loss in the subsequent SPE step, the SEC elution volume of the analyte plug of interest should not be larger than the SPE breakthrough volume (750 μL). In order to circumvent these limitations, the use of a small column containing restricted access material is currently investigated.

Overall, it can be concluded that the present system is suitable for the quantitative analysis of small peptides in CSF. Furthermore the present approach seems also very suitable for the analysis of other small molecules, such as pharmaceuticals, that will be retained longer in the SEC column than the enkephalin peptides.

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