ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
30 (2002) 1393–1401

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

www.elsevier.com/locate/jpba

Short communication

On-line coupling of SEC and RP–LC for the determination of structurally related enkephalins in cerebrospinal fluid

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

[a] *Department of Biomedical Analysis, Faculty of Pharmaceutical Sciences, Utrecht University, PO Box 80082, Sorbonnelaan 16, 3584 CA Utrecht, The Netherlands*

[b] *Protein Separations Group, Division of Chemistry, Free University Amsterdam, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands*

[c] *Department of Pharmaceutics, N.V. Organon, Molenstraat 110, PO Box 20, 5340 BH Oss, The Netherlands*

Received 15 May 2002; received in revised form 20 June 2002; accepted 4 July 2002

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 the recently reported on-line coupled size exclusion chromatography (SEC) and reversed phase liquid chromatography (RP–LC) separation system for the quantitation of structural related peptides in biological matrices, as demonstrated for a number of enkephalins in CSF. 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 (LOQ) 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 LOQ cannot be improved by increasing the sample amount, because of interference of other endogenous components of the CSF. 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.

Keywords: Cerebro-spinal fluid; Enkephalins; Multidimensional separations; On-line coupling; Reversed phase liquid chromatography; Size exclusion chromatography

1. Introduction

Peptides are becoming more and more important as drug substances in the treatment of various diseases. Generally, these peptides are active in

* Corresponding author. Tel.: +31-30-253-6952; fax: +31-30-253-5180

E-mail address: w.j.m.underberg@pharm.uu.nl (W.J.M. Underberg).

0731-7085/02/$ - see front matter
PII: S0731-7085(02)00434-X

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

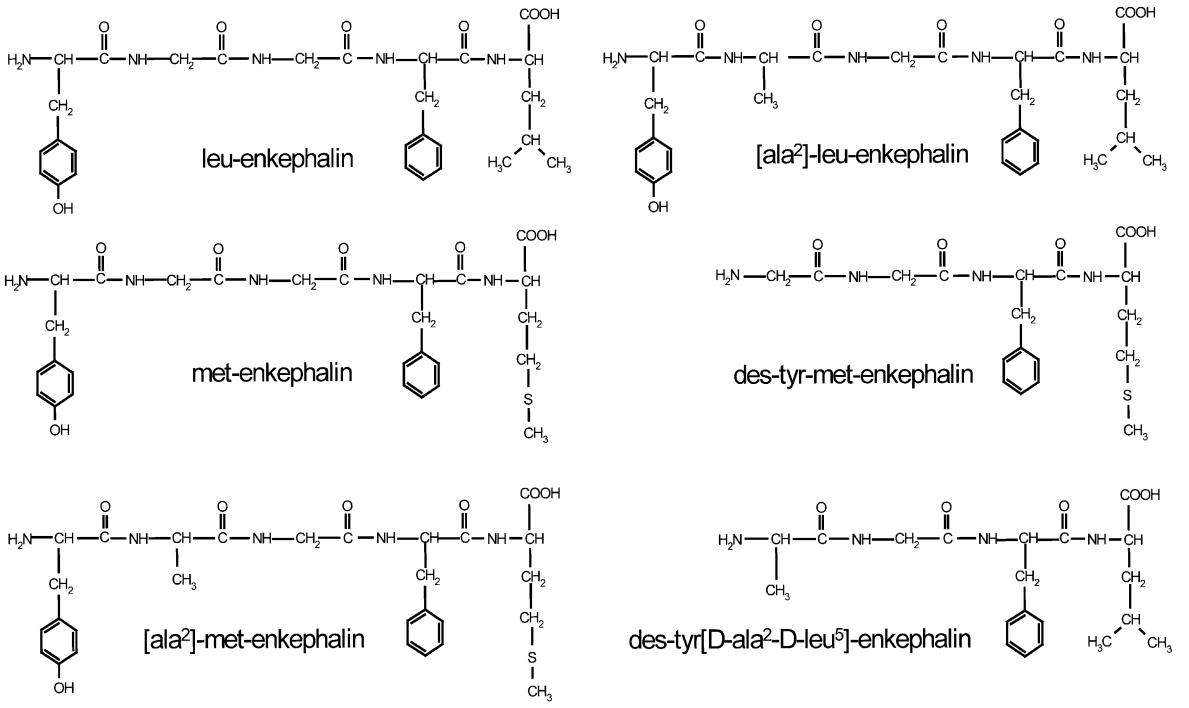

Fig. 1. Structures of the enkephalins.

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 (Fig. 1) are chosen. Special attention has also been paid to the validation of the system.

2. Experimental

2.1. 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). CSF was donated by the St. Anthonius Hospital (Nieuwegein, The Netherlands).

2.2. Instrumentation, chromatographic conditions and procedures

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

2.2.1. SEC-system

The first dimension (SEC) consists of a 4.6 mm i.d. $\times$ 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

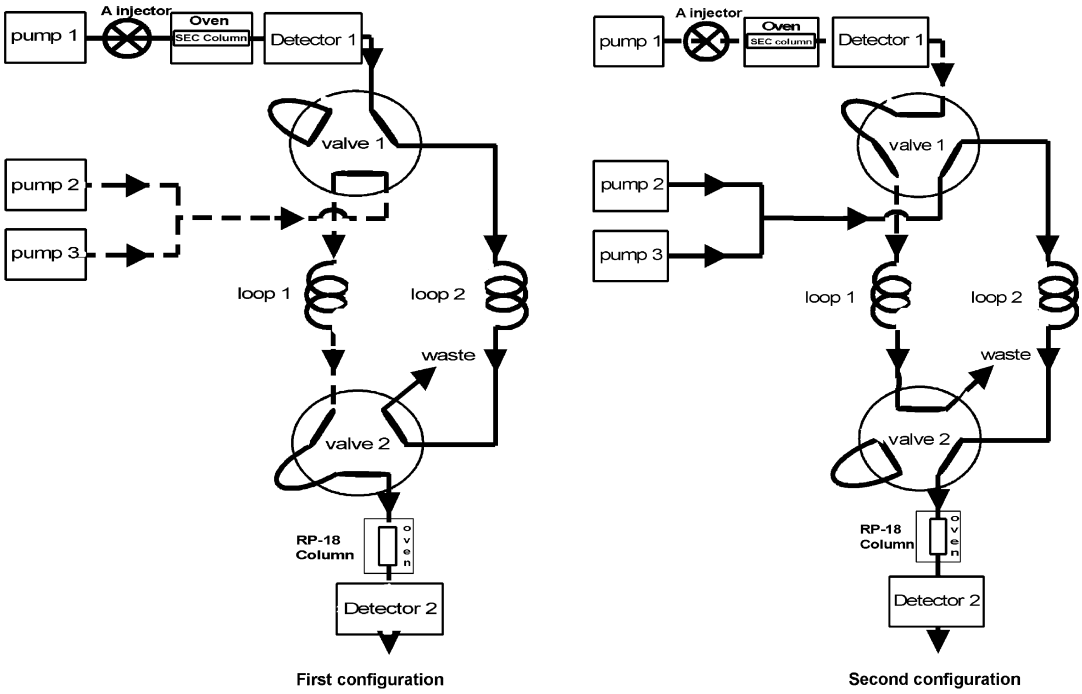

Fig. 2. Instrumental set up of the coupled SEC–LC system.

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.

2.3. 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.

2.4. 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.

2.5. 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 preconcentrated 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.

2.6. 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, 0.02 M and a blank.

Table 1
The relationship between the imipramine HCl concentration and the degradation rate, expressed by $t^{1/2}$ 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

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.

3.1. 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 1). 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 h seems acceptable,

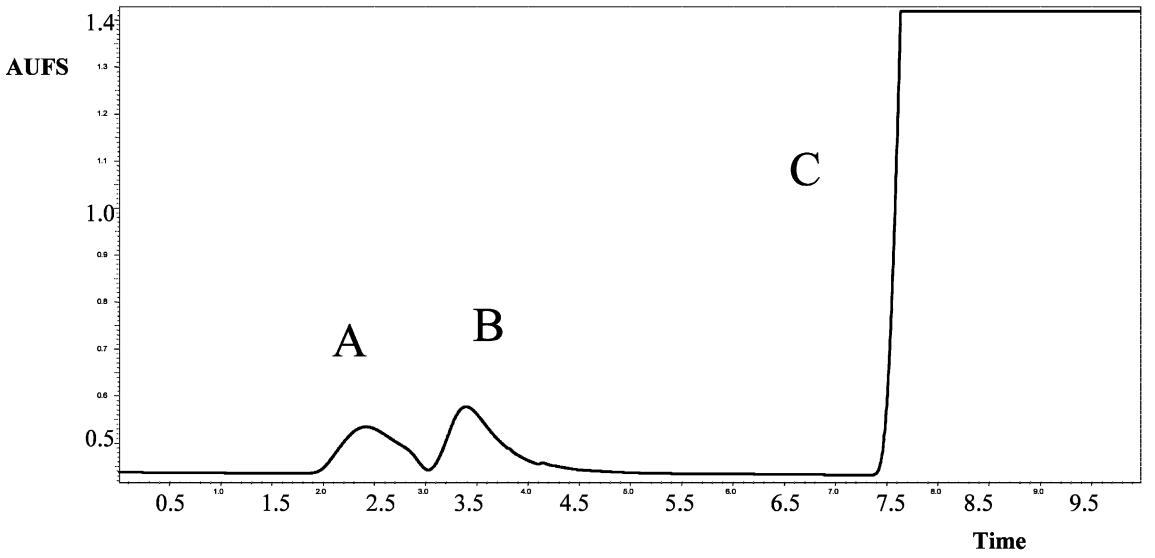

Fig. 3. Chromatogram of the SEC-separation. (A) large proteins. (B) Small peptides (enkephalins). (C) Imipramine HCl.

consequently, the half-life ($t1/2$) value has to exceed 70 h.

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 °C. 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 5 days. Table 1 displays the influence of the concentration of imipramine HCl on the degradation rate, expressed by the half-life ($t1/2$) 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.

3.2. 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

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

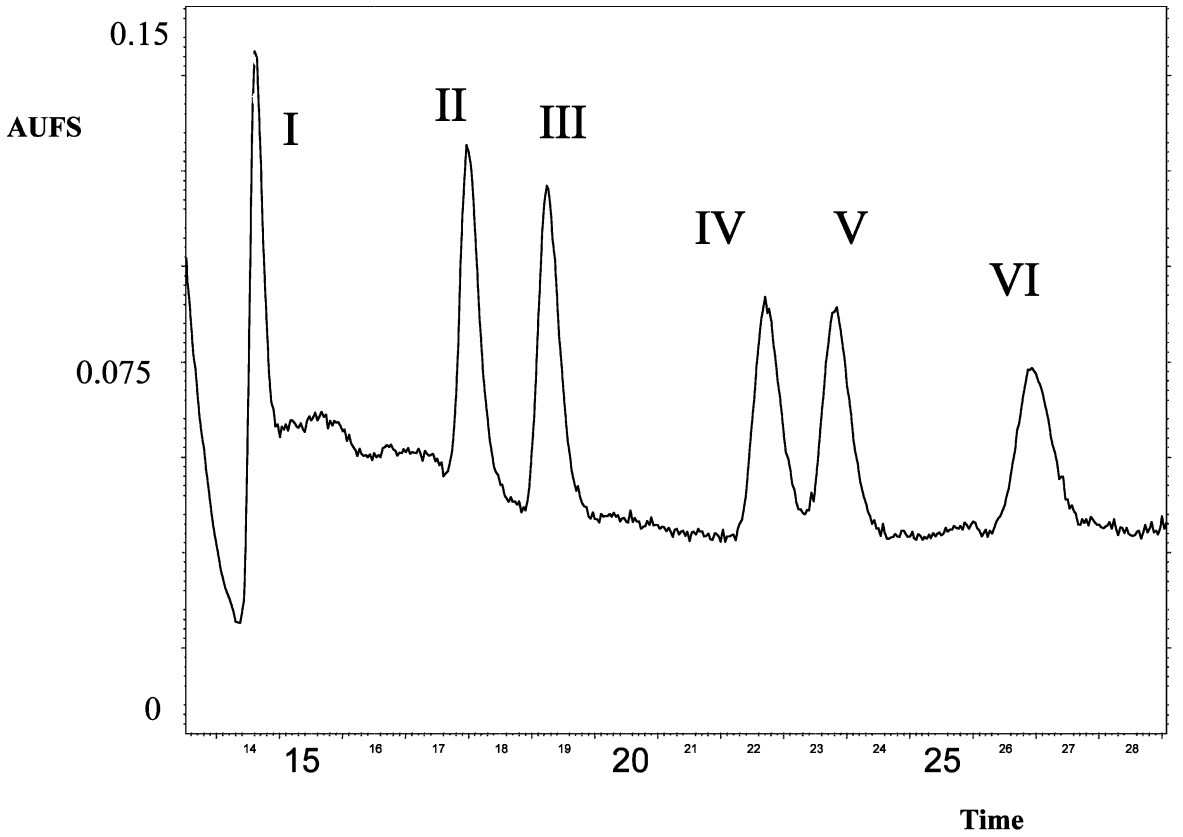

Fig. 4. Chromatogram of the separation between the enkephalins (5 μg/ml each) after injection of 10 μl with the SEC–RP–LC system. (I) Des-tyr-met-enkephalin. (II) Met-enkephalin. (III) [Ala^2]-met-enkephalin. (IV) Leu-enkephalin. (V) Des-tyr[D-ala^2-D-leu^5]-enkephalin. (VI) [Ala^2]-leu-enkephalin.

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 dimen-

Table 2
Linearity: equation, S.D. and correlation coefficient

2–80 μg/ml, $n=17$	$Y=(a\pm\text{S.D.}a)\ X+b\pm\text{S.D.}b$	R^2
Met-enkephalin	$Y=(31737\pm828)\ X-15\,010\pm25\,555$	0.99
Leu-enkephalin	$Y=(27330\pm677)\ X-1180\pm20\,902$	0.99
Des-tyr[D-ala^2-D-leu^5]-enkephalin	$Y=(28\,428\pm1124)\ X-51\,522\pm34\,6791$	0.98
[Ala^2]-leu-enkephalin	$Y=(22\,911\pm860)\ X+18\,973\pm26\,549$	0.98
[Ala^2]-met-enkephalin	$Y=(34\,616\pm1082)\ X-30\,983\pm33\,391$	0.99
Des-tyr-met-enkephalin	$Y=(47\,145\pm2595)\ X-233\,766\pm82\,513$	0.96

Table 3
Intraday and interday precision of the system

	Interday 40 μg/ml R.S.D. (%, $n = 5$)	Interday 20 μg/ml R.S.D. (%, $n = 5$)	Intraday 40 μg/ml R.S.D. (%, $n = 5$)	Intraday 20 μg/ml R.S.D. (%, $n = 5$)
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

sion, including the removal of the large excess amount of imipramine HCL. Although the shape of the peak of des-tyr-met-enkephalin (Fig. 2, I) is influenced by the preceding negative signal it does not influence significantly the quantitation of this compound [24].

3.3. 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 2. 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 3. 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 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.

References

[1] M.A. Moseley, L.J. Deterding, K.B. Tomer, J.W. Jorgenson, Anal. Chem. 63 (1991) 109–114.
[2] G.J. Opiteck, S.M. Ramirez, J.W. Jorgenson, M.A. Moseley, Anal. Biochem. 258 (1998) 349–361.
[3] M.M. Bushey, J.W. Jorgenson, Anal. Chem. 62 (1990) 978–984.
[4] M.M. Bushey, J.W. Jorgenson, Anal. Chem. 62 (1990) 161–167.
[5] E.L. Johnson, R. Gloor, R.E. Majors, J. Chromatogr. 149 (1978) 571–585.
[6] W.M. Mück, J.D. Henion, J. Chromatogr. B 495 (1998) 41–59.
[7] E. Ban, D. Kim, E.A. Yoo, Y.S. Yoo, Anal. Sci. 13 (Suppl.) (1997) 489–492.
[8] H.G. Lee, J.L. Tseng, R.R. Becklin, D.M. Desiderio, Anal. Biochem. 229 (1995) 188–197.
[9] J.C. Giddings, J. High Res. Chrom. 10 (1987) 319–323.
[10] T. Okutani, S. Morita, A. Sakuragawa, Anal. Sci. 16 (2000) 531–533.
[11] S. Hartter, H. Weigmann, C. Hiemke, J. Chromatogr. B 740 (2000) 135–140.
[12] C. Mislanova, A. Stefancova, J. Oravcova, J. Horecky, T. Trnovec, W. Lindner, J. Chromatogr. B 739 (2000) 151–161.
[13] L.E. Vera-Avila, A.M. Nunez-Gaytan, M.R. Covarrubias-Herrara, Int. J. Environ. Anal. Chem. 71 (1998) 147–164.
[14] K.A. Sagar, M.R. Smyth, J. Pharm. Biomed. Anal. 21 (1999) 383–392.
[15] P. Guo, Z.W. Li, T. Li, X.M. Wang, F. Li, Biomed. Chromatogr. 13 (1999) 61–64.

[16] J. Henion, E. Brewer, G. Rule, Anal. Chem. 70 (1998) 650A–656A.
[17] K. Takezawa, M. Tsunoda, P. Prodos, K. Murayama, T. Santa, K. Imai, Chromatography 19 (1998) 390–391.
[18] G.J. Opiteck, J.W. Jorgenson, R.J. Anderegg, Anal. Chem. 69 (1997) 2283–2291.
[19] J.V. Amari, I. Mazsaroff, J. Chromatogr. A 729 (1996) 113–124.
[20] A.W. Moore, J.W. Jorgenson, Anal. Chem. 67 (1995) 3456–3463.
[21] M. Stromqvist, J. Chromatogr. A 667 (1994) 304–310.
[22] C.T. Mant, T.W. Lorne-Burke, N.E. Zhou, J.M.R. Parker, R.S. Hodges, J. Chromatogr. 485 (1989) 365–382.
[23] R.M. Kamp, A. Bosserhoff, D. Kamp, B. Wittmann-Liebold, J. Chromatogr. 317 (1984) 181–192.
[24] K. Wagner, K. Racaityte, K.K. Unger, T. Miliotis, L.E. Edholm, R. Bischoff, G. Marko-Varga, J. Chromatogr. A 893 (2000) 293–305.
[25] T. Stroink, G. Wiese, H. Lingeman, A. Bult, W.J.M. Underberg, Anal. Chim. Act. 444 (2001) 193–203.
[26] H. Chen, F. Noble, P. Coric, M. Fournie-Zalusky, B.P. Royues, Proc. Natl. Acad. Sci. USA 95 (1998) 12028–12033.
[27] J.M. de Gandarias, O. Casis, A. Varona, M. Gallego, J. Irazusta, L. Casis, Life Sci. 61 (1997) PL321–PL326.
[28] M. Gallego, L. Casis, O. Casis, Eur. J. Pharmacol. 360 (1998) 113–116.
[29] M. Marini, A. Urbani, E. Trani, L. Bongiorno, L. Giorgio Roda, Peptides 18 (1997) 741–748.
[30] P. Roumeliotis, K.K. Unger, J. Chromatogr. 218 (1981) 535–546.