Journal of Chromatography, 511 (1990) 137–145
Elsevier Science Publishers B.V., Amsterdam

CHROM. 22 321

Bioanalysis of the peptide des-enkephalin-γ-endorphin

On-line sample pretreatment using membrane dialysis and solid-phase isolation

D. S. STEGEHUIS, U. R. TJADEN* and J. VAN DER GREEF
Division of Analytical Chemistry, Center for Bio-Pharmaceutical Sciences, University of Leiden, P.O. Box 9502, 2300 RA Leiden (The Netherlands)
(First received July 18th, 1989; revised manuscript received December 18th, 1989)

ABSTRACT

Des-enkephalin-γ-endorphin is a neuroleptic endogenous peptide that is active in the central nervous system in extremely low concentrations. The pharmacokinetics of this peptide could not be studied in detail as a bioanalytical method for determining endogenous levels of this peptide in biological matrices was not available. Liquid chromatography with fluorescence reaction detection in principle offers sufficient sensitivity for this application, provided that a selective sample pretreatment can be performed. The development of a pretreatment method for plasma samples is described. After protein precipitation with trichloroacetic acid, high-molecular-weight compounds are removed using on-line continuous-flow dialysis. After dialysis, polar low-molecular-weight compounds, including those containing amino functions, are removed by solid-phase isolation, while simultaneously the analyte is concentrated. By means of valve switching the pretreatment system is coupled on-line to the liquid chromatographic system. With the developed system it is possible to determine des-enkephalin-γ-endorphin in plasma in the range 10–100 ng/ml.

INTRODUCTION

Des-enkephalin-γ-endorphin (DEγE) or β-endorphin(6–17) is an endogenous non-opoid γ-type endorphin and may be considered as a neuroleptic-like compound in the central nervous system with antipsychotic activity in schizophrenic patients[1,2]. It is active in very low concentrations. The uptake by the brain of this neuropeptide appears to be relatively low; a maximum of *ca.* 2% of an intravenously administered dose has been reported[3]. The disappearance of DEγE from plasma after an intravenous injection can be described by a one-compartment model and the resulting half-life is about 2 min. Degradation products of DEγE in plasma are formed by carboxypeptidase and aminopeptidase activity[3,4]. The main metabolites are β-en-

0021-9673/90/$03.50

dorphin(7–17) and β-endorphin(8–17)[5]. The exact working mechanism of DEγE cannot be investigated until a bioanalytical method is available for determining endogenous levels of this peptide in biological matrices.

For more detailed pharmacokinetic studies there is a need for a selective and sensitive bioanalytical method. Plasma samples contain low concentrations of the analyte peptide in combination with high concentrations of interfering compounds that are similar to the analyte, that degrade it and interfere with the bioanalysis. Plasma levels down to the sub-ng/ml range have to be measured. A method based on post-column derivatization with *o*-phthaldialdehyde (OPA) and fluorescence detection is in principle sensitive enough for this application. However, a selective pretreatment method is required, which can only be achieved by combining a number of pretreatment steps. So far no bioanalysis permitting the determination of DEγE in plasma in the low-ng/ml range has been described, apart from a radioimmuno assay method[3].

This paper describes a method that uses deproteination of the sample with trichloroacetic acid in order to stabilize the samples, continuous-flow dialysis to remove interfering compounds with a molecular weight above 10 kDa and an on-line solid-phase isolation (SPI) on XAD-2 between the dialysis and the liquid chromatographic (LC) step to concentrate the analyte. All manipulations can be automated, except the stabilization step, which has to be performed as soon as possible after the sample is taken. The LC system consists of a reversed-phase C_{18} column and an acetonitrile and phosphate buffer gradient.

EXPERIMENTAL

Equipment

The continuous-flow system consisted of a Skalar (Breda, The Netherlands) Model 1 autosampler with solvent flux tubing and a Skalar Model 202 peristaltic pump. The polystyrene autosampler tubes had a volume of 3.5 ml. The dialysis was performed with a Skalar Model 5275 70-cm Perspex dialysis block equipped with a Type C cellulose membrane. The precolumn was a stainless-steel cartridge (3.0 cm × 2.0 mm I.D.) packed manually with XAD-2 in methanol under reduced pressure. The valve-switching system was a MUST (Spark Holland, Emmen, The Netherlands) equipped with two six-port switching valves (Model 7001; Rheodyne, Berkeley, CA, U.S.A.).

Injections were performed with a Rheodyne fixed-volume (100 μl) Model 7125 injection valve. The LC gradient system consisted of two dual-piston high-pressure pumps (Spectroflow 400; Kratos, Ramsey, NJ, U.S.A.) controlled by an SF 450 programmer (Kratos). A laboratory-made hogh-pressure mixing device with an internal volume of about 400 μl and equipped with a stirring magnet was applied to mix the effluent of the two pumps. The analytical column was a glass cartridge (10 cm × 3.0 mm I.D.) packed with Chromospher C_{18} (5-μm particles) (Chrompack, Middelburg, The Netherlands). The post-column derivatization device consisted of a Model P-35 high-pressure pump (Pharmacia, Uppsala, Sweden). A stainless-steel reaction coil (4 μm I.D.) was spirally wound with O.D. *ca.* 2 cm and an internal volume of *ca.* 0.6 ml. A stainless-steel dead-volume mixing device (Upchurch Scientific, Oak Harbor, WA, U.S.A.) was applied for mixing of the effluent and the derivatization

reagent. Fluorescence detection was performed with a Perkin-Elmer (Beaconsfield, U.K.) LS-4 detector using an excitation wavelength of 334 nm, an emission wavelength of 455 nm and a slit width of 5 nm. Chromatograms were recorded with an electronic integrator (Model C-R3A; Shimadzu, Kyoto, Japan).

Materials

DEγE was donated by Organon International (Oss, The Netherlands). Trichloroacetic acid was obtained from Baker Chemicals (Deventer, The Netherlands), mercaptoethanol and *o*-phthaldialdehyde from Fluka (Buchs, Switserland) and LC-grade acetonitrile from Rathburn (Walkerburn, U.K.). The phosphate buffers were composed of different volumes of 0.01 mol/l phosphoric acid and 0.01 mol/l disodium hydrogenphosphate (Brocacef, Maarsen, The Netherlands). The borate buffers were mixtures of 0.1 mol/l sodium tetraborate and 0.01 mol/l sodium hydroxide (Merck, Darmstadt, F.R.G.). Deionized water (Milli-Q water purification system; Millipore, Bedford, MA, U.S.A.) and capped polopropylene vials (Greiner, Alphen a/d Rijn, The Netherlands) were used for all peptide-containing solutions. Amberlite XAD-2 (Rohm and Haas, Philadelphia, PA, U.S.A.) with a particle size range of 20–30 μm was applied. Prefabricated normal-phase, reversed-phase and ion-exchange precolumns (Analytichem, Harbor City, CA, U.S.A.) were used.

Protein precipitation

The protein precipitation experiments were performed with 1-ml plasma samples spiked with 1 ng/ml of the analyte. The plasma sample was mixed with 2 ml of methanol, ethanol or acetonitrile or with 300 μl of trichloroacetic acid or perchloric acid. After vortex mixing for 10 s the mixtures were centrifuged for 10 min (2500 *g*) and subsequently the supernatant was transferred to the polypropylene autosampler vials and analysed by the described system. Some samples were analysed immediately and others after 3 h or after 3 days. The deproteinated samples were stored after centrifugation at −10°C.

On-line dialysis

The on-line dialysis experiments were performed with the deproteinated plasma samples. The pH of the supernatant (650 μl) was adjusted to 5.7 with 350 μl of 0.1 mol/l borate buffer in the autosampler vials. The whole sample was transported to the dialysis block. The inside diameters of the tubing were chosen to result in a flow of the donor (sample) stream of 0.32 ml/min and with a flow-rate of 0.42 ml/min of the acceptor (water) stream. The donor stream was segmented with air at 0.16 ml/min. The system was used in the countercurrent mode. In this way the dialysis took about 15 min. The acceptor stream was loaded on the XAD-2 column in about 3 min.

Solid-phase isolation

XAD-2 was used in an on-line trapping and isolation column. The column was used as an interface between the dialysis system and the LC system. By switching the MUST unit the sample was transferred from one system to the other.

Chromatographic system and detection

Chromatography was performed with a C_{18} analytical column in combination

with gradient elution. Eluent A was 0.05 mol/l phosphate buffer (pH 2.4) and eluent B was 0.05 mol/l phosphate buffer (pH 2.4)–acetonitrile (50:50, v/v). A step gradient was applied: 2 min 15% B, 2 min 40% B, 6 min 75% B and finally 2 min 15% B. With 15% B the analyte did not elute from the XAD-2; with 40% B the analyte was eluted from the XAD-2 to the analytical column.

The reagent solution for the post-column OPA derivatization consisted of 2 ml of borate buffer (pH 9.5), 9 mg of OPA and 300 μl of mercaptoethanol. The flow in the post-chromatographic derivatization unit was 0.75 ml/min and was established by an eluent flow-rate of 0.5 ml/min and a reagent flow-rate of 0.25 ml/min.

RESULTS AND DISCUSSION

The peptide DEγE is unstable in enzyme-containing matrices. Therefore, the first priority is the enzyme deactivation by deproteination in order to stabilize the analyte concentration in the sample. Another important aspect during the bioanalytical procedure is to avoid the use of glass vessels because DEγE is adsorbed to polar surfaces such as glass.

The molecular weight of DEγE is 1304 so it can be separated from all high-molecular-weight compounds by ultrafiltration or dialysis. In this study, the possibilities of on-line continuous-flow dialysis were investigated[6]. The advantages of this method are an absolute cut-off value at molecular weight 10 000 and the fact that the method can be performed on-line, which is advantageous with respect to reproducibility and labour intensity.

DEγE possesses two primary amino functions and two carboxylic acid functions. The isoelectric point is at pH 5.7. These characteristics combined with its polarity and hydrophobicity make it possible to separate the peptide from the remaining low-molecular-weight compounds using SPI and LC. As the post-column OPA derivatization is specific for primary amino-containing compounds, the sample has to be purified to remove these as much as possible. With respect to the strong dilution that takes place during the dialysis, the most suitable purfication step is a solid-phase isolation using a reversed-phase material such as XAD-2. The preconcentration column can be installed in a valve switching unit and the sample can be loaded on the XAD-2 and introduced automatically into the LC system where it is eluted by an acetonitrile gradient[7].

Sample stabilization

Deproteination of 1 ml of plasma by adding 2 ml of an organic solvent such as methanol, ethanol and acetonitrile appeared not to be sufficient for complete inhibition of the enzyme activity. Even immediately after such a protein precipitation, degradation of DEγE could be observed. A few minutes after deproteination the recovery had been reduced to about 5%, and decreased further as a function of time.

Precipitation with either 1 mol/l perchloric acid (PCA) or 1 mol/l trichloroacetic acid (TCA) appeared to be superior to the former method. With neither precipitating agent was any analyte degradation observed, but addition of TCA resulted in more precipitate while the supernatant was more transparent. The amount of interfering compounds was minimal after deproteination with TCA.

A sample of 1 ml of plasma deproteinated by adding 300 μl of 1 mol/l TCA was

stable after 3 h and even after 3 days no degradation of the analyte was observed. TCA has only a limited buffer capacity, so pH adjustments are facile. In addition, there will be no problem with respect to the compatibility with the dialysis, which should be a problem after deproteination with organic solvents (the cellulose membrane dissolves in organic solvents). The recovery of the analyte after the precipitation with TCA is about 85%. The loss is probably due to inclusion in the precipitate and adsorption on precipitated matrix compounds. This was confirmed by assaying samples that were spiked after enzyme deactivation, but before the vortex mixing and centrifugation. Possible decreases in recovery appeared to be due to precipitation and adsorption and not to degradation.

On-line dialysis

The system was equipped with a cellulose membrane with a molecular mass resistance of 10 000 Da. The recovery of the analyte is determined by the contact time between the donor and the acceptor stream (the actual dialysis time). This contact time depends on the flow of the donor and acceptor stream and whether the concurrent or the countercurrent mode is applied. In the concurrent mode the donor and acceptor streams flow in the same direction and, assuming equal flow-rates, equilibrium can be obtained between the donor and acceptor streams. After an infinite dialysis time the theoretical maximum recovery of 50% can be achieved. In the countercurrent mode the donor and acceptor streams flow in opposite directions. In this way the dialysis can be exhausting, resulting in a recovery of 100%. Unfortunately, increasing the recovery of the analyte by prolonging the contact time between the donor and acceptor streams does not result in an improved selectivity, as the ratio between the analyte and background is constant at all recoveries.

Air segmentation of the donor stream is performed to avoid contamination of subsequent samples, to limit sample dispersion and to optimize the flow pattern towards the membrane by convective mixing in each liquid compartment. This is not of importance for the acceptor stream because it is concentrated after the dialysis on the trapping column.

The influence of the ionic strength and the pH on the dialysis was investigated. The best results were obtained when both the donor and the acceptor streams had minimum ionic strength. If a significant difference in ionic strength exists between the donor and acceptor streams this results in a water flow through the membrane, resulting in pressure irregularities which influence the flow and thereby the dialysis time. Buffer solutions with molarities varying from 0.001 to 0.1 were used as donor and acceptor streams while the recovery of DEγE was monitored. The influence of pH was caused by the charge of the analyte. DEγE is an ampholite with five possible charge stages. At pH < 2 it is charged 2+, at about pH 4 the charge is 1+, the isoelectric point is at pH 5.7, at about pH 8 it is charged 1− and at pH > 11 it is charged 2−. Because there are polarizable functions present on the membrane the dialysis should be performed at the isoelectric point of the analyte (pH 5.7). In this case adsorptions and electric repulsions will be minimized (Fig. 1). This was done with buffer solutions of different pH so that the recovery of DEγE could be determined at all its possible charge stages. At the optimum solvent composition the concurrent and countercurrent modes were compared just like segmented and unsegmented donor and acceptor streams. Although dialysis is normally a time-consuming procedure, on-line dialysis applying

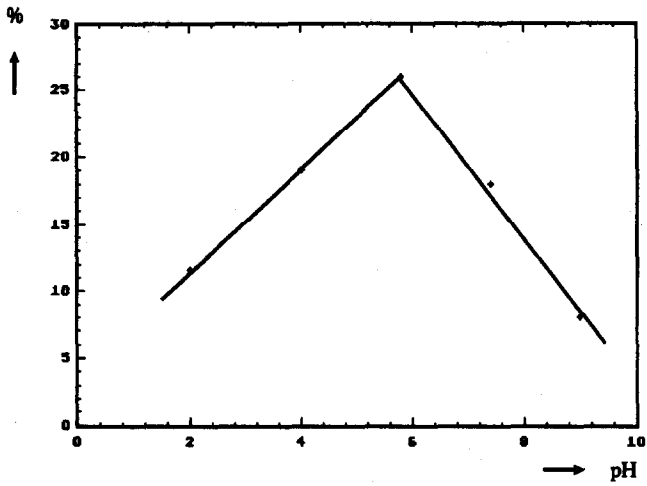

Fig. 1. Recovery of DEγE as a function of the pH of the donor stream in the continuous-flow dialysis system.

a continuous-flow system permits the reproducible removal of proteins in about 15 min with a recovery of the peptide of 25%, applying optimum conditions, *i.e.*, use of the countercurrent mode, an air-segmented donor stream, minimum ionic strength of the donor and acceptor streams and dialysis at the isoelectric point of the analyte peptide (pH 5.7). The overall recovery, determined by assaying a standard solution of DEγE, by performing the complete sample pretreatment, *i.e.*, on-line dialysis, solid-phase isolation and heart-cutting of the gradient run, was 25.0 ± 0.9%, independent of the concentration range studied (10 ng/ml–5 μg/ml). When the dialysis took place at a sample pH of 2, caused by addition of TCA, the recovery was only 12%.

Solid-phase isolation

In principle, on-line dialysis is not compatible with LC because of the relatively large dilution inherent to on-line dialysis. Therefore, preconcentration of the sample should be executed before it can be analysed using LC. In fact, the solid-phase isolation is the interface between the (strongly diluting) dialysis and the (small injection volume demanding) chromatography.

Our objective was to combine precolumn concentration of the dialysed sample and removal of most of the low-molecular-weight compounds containing a primary amino group such as amino acids and biogenic amines that can interfere with the final detection. In order to use the precolumn for this purpose the analyte should be adsorbed on the sorbent, whereafter most of the interfering compounds are eluted from the column and finally the analyte is selectively eluted from the precolumn. The most important parameters are the breakthrough volume (this volume should be large enough to concentrate the sample after the dialysis and to wash the column without losing the analyte) and the elution volume (the amount of solvent needed to elute the analyte). The breakthrough volume should be as large as possible while the elution volume should be minimal.

To find the most suitable column material for the solid-phase isolation a number

of normal-phase, reversed-phase and ion-exchange materials were investigated. The polymeric neutral resin XAD-2 appeared to be most suitable in combination with the on-line dialysis system, as the capacity ratio of the analyte on the precolumn is sufficiently high ($k' > 10$) to concentrate the aqueous 10 ml of the dialysed sample. The capacity factor of DEγE was determined on this material by loading an aqueous sample of DEγE on the XAD-2 column and eluting it with different percentages of acetonitrile (Fig. 2). The elution of the analyte could be done in a small fraction of about 0.5 ml with a mixture of phosphate buffer containing at least 15% of acetonitrile (Fig. 2). This fraction was directly eluted to the analytical column. The hydrophobic reversed-phase material XAD-2 in a precolumn of length 3.0 cm is able to concentrate the acceptor stream fraction of about 10 ml and to isolate the analyte from most of the amino acids without influencing the elution profile.

Total system

Apart from the off-line enzyme deactivation step, which must be performed as soon as possible after blood sampling, the other steps of the sample pretreatment were combined in an on-line system (Fig. 3). Deproteinated samples are transported to the dialysis system by means of a peristaltic pump.

After removal of the macromolecules, the sample is concentrated on the XAD-2 column, where interfering low-molecular-weight compounds are removed to a considerable extent. Subsequently the analyte-containing fraction is eluted to the analytical column where it is separated from related compounds, derivatized in the post-column mode and detected by fluorescence detection. Using this method, the analysis of one sample takes about 45 min. Because the total procedure is performed in the on-line mode, the method has minimum labour requirements.

With continuous analyses every 30 min, samples can be analysed during 24 h a day. As in most continuous-flow systems the reproducibility is an advantage. At a level of 250 ng/ml DEγE in plasma the relative standard deviation (R.S.D.) was 2.7%

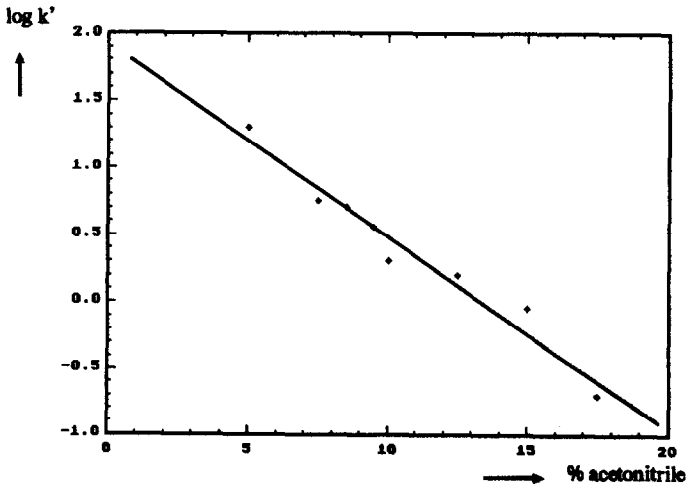

Fig. 2. Capacity ratios (k') for DEγE on the 30 mm × 2 mm I.D. XAD-2 precolumn as a function of the acetonitrile content of the mobile phase.

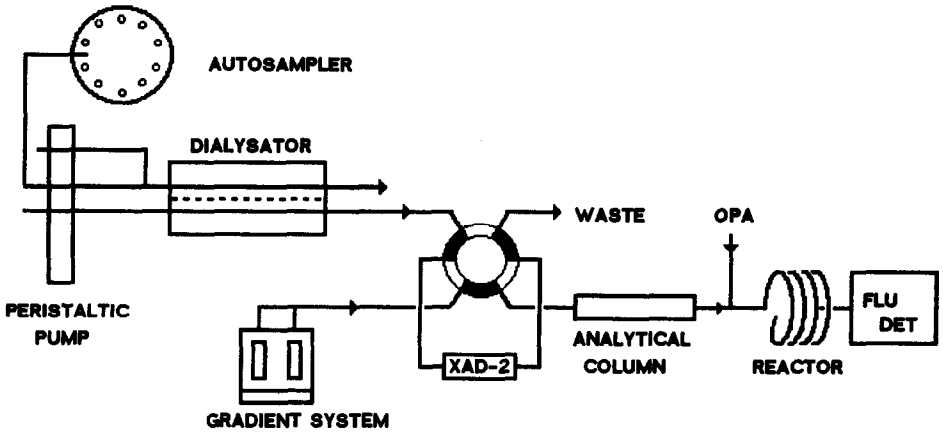

Fig. 3. Scheme of total pretreatment procedure. FLU DET = Fluorescence detector.

(n = 5), which is acceptable for this type of analysis. At lower levels (50 ng/ml DEγE) the R.S.D. was 5.9% (n = 5). Some results are shown in Fig. 4.

The large hump in the chromatograms, also in the chromatogram of an aqueous solution without DEγE (see Fig. 4A), is caused by the acetonitrile gradient, which is present even when freshly distilled acetonitrile is used. In our opinion this phenomenon is caused by the gradient elution of strongly hydrophobic compounds that are adsorbed from the mobile phase and previously injected samples, and that are in fact enriched in the so-called on-column mode. It must be emphasized that all constituents of the mobile phase were of high analytical purity and that the water used was highly purified. Fig. 4B shows the chromatogram obtained from a plasma sample spiked with

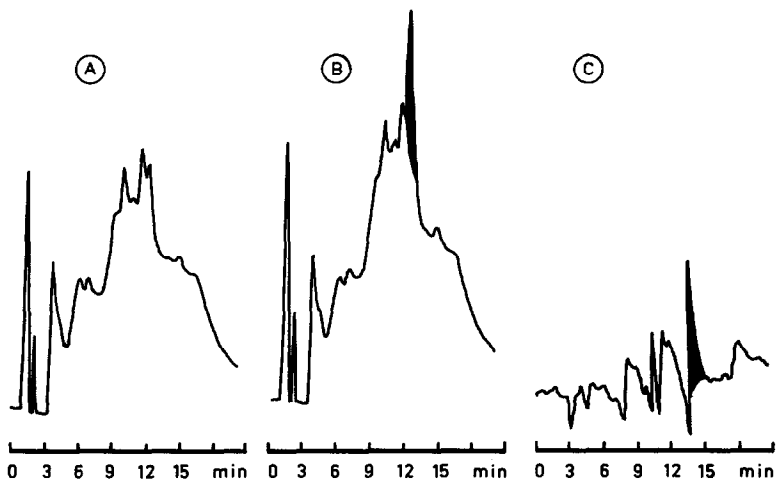

Fig. 4. Chromatograms of plasma samples treated according to the described procedure. (A) Blank plasma; (B) plasma spiked with 100 ng/ml DEγE; (C) differential chromatogram of plasma spiked with 10 ng/ml DEγE and blank plasma. Injection volume, 500 μl of plasma. For other conditions, see text.

100 ng/ml of DEγE. Apparently smaller amounts cannot be determined, but by applying electronic background subtraction with the integrator a differential trace is obtained, as shown in Fig. 4C. In this instance the blank gradient run (Fig. 4A) is subtracted from the chromatogram obtained from a plasma sample spiked with 10 ng/ml of DEγE.

In spite of the hump in the baseline, a calibration graph could be generated without any problem in the concentration range 10–500 ng/ml DEγE in plasma. A typical calibration graph, represented by the equation $y = 2.47x + 14.6$ with a correlation coefficient of 0.9991, where x and y are the concentration in ng and the peak height in arbitrary units, respectively, was generated by analysing blank plasma samples spiked in the concentration range mentioned above. In the spiked samples and blank it is shown that the method is selective enough for this concentration range (10–100 ng/ml).

CONCLUSIONS

The method described, based on continuous-flow dialysis, solid-phase isolation, LC separation and fluorescence reaction detection with OPA, permits the measurement of plasma samples containing DEγE down to 10–100 ng/ml. To lower this detection limit the selectivity of the sample pretreatment procedure would have to be increased by applying methods with greater selectivity at higher recoveries, *e.g.*, gel filtration or electrophoresis. The sensitivity of detection may then be further increased by using laser-induced fluorescence detection[8]. Further work in these directions is in progress.

REFERENCES

1 D. de Wied, *Trends Neurosci.*, 2 (1979) 79.
2 W. M. A. Verhoeven, J. M. van Ree, A. Heezius van Bentum, D. de Wied and H. M. van Praag, *Arch. Gen. Psychiatry*, 39 (1982) 648.
3 J. C. Verhoef, H. Scholtens, E. G. Vergeer and A. Witter, *Peptides*, 6 (1985) 467.
4 D. de Wied, J. M. van Ree and H. M. van Gerven, *Life Sci.*, 26 (1980) 1575.
5 P. S. L. Janssen, J. W. van Nispen, P. A. T. A. Melgers and R. L. A. E. Hamelinck, *Chromatographia*, 21 (1986) 461.
6 U. R. Tjaden, E. A. de Bruijn, R. A. M. van der Hoeven, C. Jol, J. van der Greef and H. Lingeman, *J. Chromatogr.*, 420 (1986) 53.
7 U. R. Tjaden, D. S. Stegehuis, H. J. E. M. Reeuwijk, H. Lingeman and J. van der Greef, *Analyst (London)*, 113 (1987) 171.
8 C. M. B. van den Beld, H. Lingeman, G. J. van Ringen, U. R. Tjaden and J. van der Greef, *Anal. Chim. Acta*, 205 (1988) 15.