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Bioanalysis of the neuropeptide des-enkephalin-γ-endorphin by high-performance liquid chromatography with on-line sample pretreatment using gel permeation and solid-phase isolation

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ABSTRACT

A bioanalytical method is described that allows the determination of a number of β-endorphin-related peptides. The method is based on the application of fluorescence detection after high-performance liquid chromatography followed by post-column derivatization with *o*-phthaldialdehyde. Concentrations exceeding 10–25 ng/ml could be determined by using conventional fluorescence detection, whereas lower concentrations demand the use of laser-induced fluorescence detection. The sample pretreatment includes the use of on-line gel permeation, on-line solid-phase isolation and heart cutting of a peak from reversed-phase gradient elution. The sample pretreatment procedure does not discriminate between the dodecapeptide des-enkaphalin-γ-endorphin (DEγE) and its metabolites in order to obtain similar recoveries for all components. The final chromatographic phase system is based on ion-pair formation, which permits the separation of DEγE from its metabolites and degradation products. The optimized procedure allows the determination of these peptides in plasma at concentration levels down to about 1 ng/ml, demanding a sample volume of 1 ml.

INTRODUCTION

There is growing interest in the bioanalysis of endogenous peptides owing to their pharmacological activity at low concentration at several sites in the human body. As an example, β-endorphins play an important role in the central nervous system. For a number of these peptides immunoassays have been developed [1]. Although methods based on radiochemical techniques, such as radioimmunoassays (RIA) and radioreceptor assays (RRA), allow the determination of extremely low concentrations [2,3] directly in the biological matrix, these methods may be not sufficiently selective with respect to structurally closely related metabolites and degradation products. A practical drawback of RIA and RRA is the application of radioactive labels, demanding a laboratory equipped for radioactive measurements. Non-radioactive bioanalytical methods may therefore be attractive. In addition to immunoassays, chromatographic methods such as liquid chromatography (LC) in combination with UV absorbance or fluorescence detection can be considered. The

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peptides that have been investigated in this study, however, show poor spectrophotometric and electrochemical [4] characteristics. We focused on the peptide des-enkaphalin-γ-endorphin (DEγE) and metabolites that interfere in the RIA owing to the high cross-reactivity. In order to allow the determination of these peptides at the ng/ml or even lower plasma levels, their detectability has to be improved, *e.g.*, by employing pre-or post-column derivatisation techniques [5,6]. Further, the compounds of interest have to be separated from an excess of interferents originating from the biological matrix.

The combination of high-performance liquid chromatography (HPLC), post-column derivatization and fluorescence detection has proved to be a powerful tool in the bioanalysis of peptides. Detection limits in the picogram range could be obtained in this way [7,8]. From earlier work it is known that the sample clean-up is extremely important [9], especially when laser-induced fluorescence (LIF) detection is used [10]. A sample clean-up for DEγE has been described in a previous paper [9] and consisted of a combination of on-line dialysis and solid-phase isolation. This method resulted in a determination limit for this peptide of about 10 ng/ml in plasma, although the detection limit for the *o*-phthaldialdehyde derivative of this peptide was 100 pg/ml. The discrepancy between these two limits is mainly caused by the sample clean-up procedure, which therefore has to be improved with respect to selectivity towards the biological background. Therefore, we investigated the use of gel permeation for sample pretreatment as an alternative to on-line dialysis for the removal of macromolecular compounds, because these matrix constituents will severely interfere with the analysis of peptides [11,12]. Regarding the determination of DEγE the immediate removal or deactivation of enzymes is especially important, because they cause its degradation resulting in a half-life of about 2 min [9]. Also, the plugging and modification of the chromatographic system that can be caused by these macromolecular compounds can be very disturbing when larger sample volumes (1 ml) are introduced. Even after deproteination with trichloroacetic acid to decrease enzyme activity, an unacceptable amount of relatively high-molecular mass compounds was still present in the sample. Similar selectivities are expected from gel permeation and dialysis, as both methods have in principle the same selectivity criteria, which are based on molecular size. However, a clear advantage that can be expected from gel permeation is that, in addition to selectivity towards the higher molecular mass compounds, it also offers selectivity towards the lower molecular mass range combined with additional adsorption characteristics.

EXPERIMENTAL

Chemicals

Trichloroacetic acid (TCA) was supplied by Baker Chemicals (Deventer, Netherlands). Mercaptoethanol (ME) and *o*-phthaldialdehyde (OPA) by Fluka (Buchs, Switzerland) and LC-grade acetonitrile by Rathburn (Walkerburn, UK). Synthetic human DEγE (β-endorphin 6–17; βE 6–17) and its metabolites (βE 7–17 and βE 8–17) were donated by Organon International (Oss, Netherlands). The phosphate buffers were composed of different volumes of 0.05 *M* phosphoric acid and 0.05 *M* disodium hydrogenphosphate purchased from Brocacef (Maarssen, Netherlands). The borate buffer was prepared from 0.1 *M* sodium tetraborate.

Throughout the study deionized water obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA) and capped polypropylene vials (Greiner, Alphen a/d Rijn, Netherlands) were used for all peptide-containing solutions. Amberlite XAD-2 (Rohm and Haas, Philadelphia, PA, USA) with a particle size range of 20–30 μm and Sephadex G-50 superfine (Pharmacia, Uppsala, Sweden) were used in the gel columns.

Equipment

The HPLC gradient system consisted of two dual-piston high-pressure pumps (Spectroflow 400; Kratos, Ramsey, NJ, USA) controlled by a Spectroflow SF450 programmer. A laboratory-made high-pressure mixing device with an internal volume of about 400 μl with a stirring magnet was applied. The switching system was a MUST (Spark Holland, Emmen, Netherlands) equipped with two six-port valves (Model 7010; Rheodyne, Berkely, CA, USA). Injections were performed with a fixed-volume (100 μl) injection valve (Model 7125; Rheodyne). Fluorescence detection was performed with a Perkin-Elmer (Beaconsfield, UK) LS 4 detector using an excitation wavelength of 334 nm, an emission wavelength of 455 nm and a slit width of 5 nm for both excitation and emission. The analytical column (100 × 3.0 mm I.D.) was laboratory-packed with C_{18} Nucleosil (5 μm) (Macherey, Nagel & Co., Düren, Germany). The precolumn was a stainless-steel cartridge (30 × 2.0 mm I.D.) packed with XAD-2 (20–30 μm). The post-column derivatization device consisted of a Model P 3500 high-pressure pump (Pharmacia) and a stainless-steel reaction coil (400 μm I.D.) spirally coiled with a coil diameter of about 2 cm. The internal volume was about 0.2 ml. A stainless-steel dead-volume T-piece (Upchurch Scientific, Oak Harbor, WA, USA) was used to mix the effluent and the derivatization reagent. The continuous-flow system consisted of an autosampler (Model 1000), with Solvent Flux tubing, and a peristaltic pump (Model 2002), all from Skalar (Breda, Netherlands). The polystyrene autosampler tubes had a volume of 3.5 ml (Skalar). The dimensions of the gel column cartridge (Pharmacia) were 120 × 10 mm I.D. with a volume adaptor. The glass tube was replaced by a Plexiglas tube (laboratory-made). The fraction collector was a Model 100 from Pharmacia.

The LIF system, which is described in detail elsewhere [10], has as an excitation source a water-cooled continuous-wave argon-ion gas laser (Model 2025-03; Spectra-Physics, Mountain View, CA, USA). Operating in the UV mode, the laser emits at 351.1 and 363.8 nm with a total output power of 165 mW. A rectangular flow-through cuvette (Model 176.752 QS, 22.5 μl; Hellma, Müllheim/Baden, Germany) with an optical path length of 1.5 mm is used as detector cell. Because the laser beam was focused very close to the inlet capillary, an effective cell volume of 2 μl was realized. The fluorescence light was guided to the photomultiplier tube by a liquid light guide (1000 × 5.0 mm I.D., No. 77556; Oriel, Stratford, CT, USA) equipped with a plano-convex fused-silica lens (diameter 11 mm, focal length 19 mm; No. 41210) at each end. At the detector side, the light was collected by a plano-convex lens (as above) and directed into an interference filter (Oriel, Stratford, CT, USA). The fluorescence was detected by a blue–green-sensitive photomultiplier tube (PMT) (No. 9635B; Thorn EMI, Ruislip, Middlesex, UK) operating at 600 V, which was generated by a high-voltage supply (Model 244; Keithley Instruments, Cleveland, OH, USA). The signal was processed by a current amplifier (Model 427; Keithley Instruments).

Pretreatment

Plasma samples were obtained from a pharmacokinetic study with healthy volunteers in which the samples should be assayed by an RIA method. Because this method does not allow the presence of TCA, the samples were immediately frozen after sampling. These plasma samples were treated individually, because of the limited stability of DEγE in plasma. Spiking of the calibration samples was also applied one sample after another. The samples were defrosted individually and immediately deproteinated by adding 300 μl of 1 *M* TCA per millilitre of plasma. After deproteination, the samples were whirl-mixed for 3 s and centrifuged for 10 min at 1000 *g*. The supernatant (about 1 ml) was decanted and placed in the autosampler.

The whole sample was loaded onto the gel permeation column at a flow-rate of 0.3 ml/min. The fraction from 25 to 35 min was heart-cut (fraction I). This whole fraction (corresponding to 3 ml) was reconcentrated on an XAD-2 column with a flow-rate of 0.6 ml/min. After about 5 min, the XAD-2 columm was connected in series with the analytical column of the gradient system by switching valve 1 (Fig. 1). The gradient profile started for 5 min with 100% aqueous mobile phase and then 5 min at 15% acetonitrile, followed by 5 min at 27.5% acetonitrile. Between 15 and 16 min after starting the gradient, a second heart-cut (fraction II) was made by switching valve 2 (Fig. 1). This fraction had a volume of 0.5 ml.

Chromatography

For conventional fluorescence detection and LIF detection, two different chromatographic systems were used. Conventional fluorescence detection was applied for monitoring the chromatographic system belonging to the sample pretreatment part using a C_{18} analytical column (Nucleosil C_{18} 5 μm; 100 $\times$ 3.0 mm I.D.) with a mobile phase consisting of phosphate buffer (0.05 *M*, pH 2.4)–acetonitrile (4:1, v/v).

The LIF detector was used in the final step, where separation and determina-

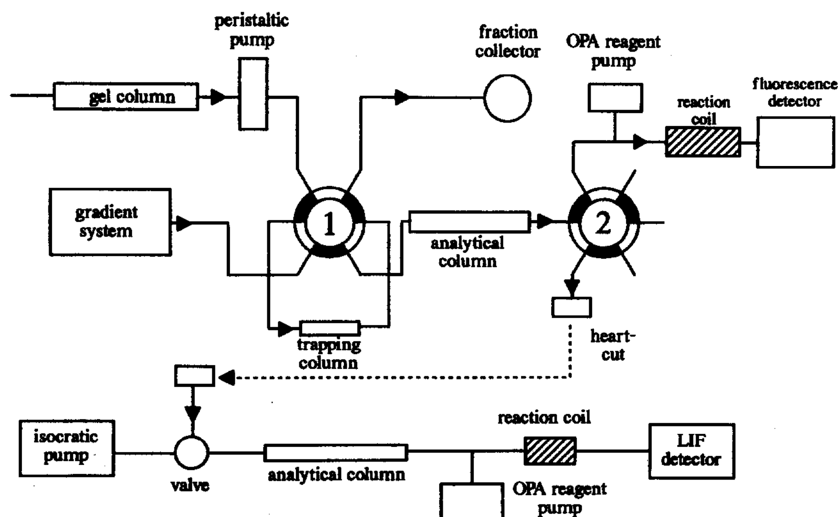

Fig. 1. Scheme of analytical system.

tion of DEγE and its metabolites took place. The chromatographic system for the LIF detector consisted of a C_{18} analytical column (see above) with a mobile phase consisting of phosphate buffer (0.01 *M*, pH 2.4)–acetonitrile–22.5 m*M* octanesulphonate (76.8:22.7:0.5, v/v/v). Two different injection methods were used. In the fluorescence detection system, the sample was diluted to an acetonitrile concentration below 10% and loaded onto an XAD-2 column. By switching valve 1 (Fig. 1), the sample was introduced into the HPLC system. In the LIF detection system, an injection loop of 1.5 ml was used, and the sample was diluted to 1.0 ml with water. After injection of the sample on the octanesulphonate system, on-column concentration took place.

Detection

Both conventional fluorescence and LIF detection, were applied, both in combination with post-column OPA derivatization. The conventional fluorescence detection system was used for the daily screening of concentrations above 10–25 ng/ml. Below this concentration, LIF detection was required to determine DEγE. In both systems, the post-column reagent was added at a flow-rate of 0.25 ml/min via a T-piece. Derivatization took place in a reaction coil with a reaction time of 15 s. The reagent solution contained 9.0 mg of OPA and 260 μl of ME in 200 ml of 0.1 *M* borate buffer (pH 9.5).

RESULTS AND DISCUSSION

In an earlier paper [9], a method was described in which on-line dialysis was used in the sample clean-up for plasma samples containing DEγE. On-line dialysis allowed the complete removal of compounds with a molecular mass above 10 000 dalton, but is not selective for plasma components with a low molecular mass. Furthermore, recoveries were relatively low (about 25%) and dependent on the dialysis time. In order to improve this method, we investigated gel permeation as a sample pretreatment technique. Gel permeation is often used as a method for separations based on molecular mass distribution [13], for example, for protein analysis [14–16] or analysis of lower molecular mass biopolymers such as peptides [17]. For gel permeation, Sephadex G-50 was chosen, because DEγE has a molecular mass of 1304 dalton and the fractionation range of this gel is between 1500 and 30 000 dalton. In principle, DEγE should be eluted within approximately one column volume but in practice is was about three void volumes. The elution profile of DEγE is given in Fig. 2. The retention volume indicates that retention is based on a combination of mechanisms. Separation of the analyte from larger molecular compounds occurs by a size-exclusion mechanism. The occurrence of capacity factors larger than the theoretical value for gel permeation implies that, in addition to size exclusion, adsorption phenomena also contribute to the retention of DEγE. The polar surface of the Sephadex stationary phase, made by cross-linking dextranes with epichlorohydrin, probably causes adsorption of the polar peptides. A similar phenomenon has been described by Schoots and Cramers [18], who compared dialysis with gel permeation for "middle molecules" with molecular masses between 300 and 1500 dalton. Because the contribution of adsorption phenomena to the retention of DEγE was very favourable regarding the selectivity of the system, no attempts were made to suppress adsorption,

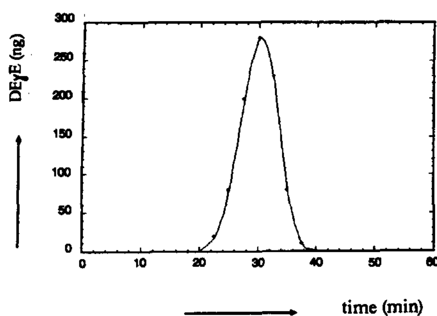

Fig. 2. Elution profile of DEγE from the Sephadex G-50 column. A 1-ml volume of solution containing 1 μg of DEγE was placed on the gel column. Fractions were analysed using conventional fluorescence detection (see text).

e.g., by adding a strong buffer to the mobile phase. DEγE also strongly adsorbs on glass surfaces; therefore, the Sephadex gel was installed in a Plexiglas tube instead of the glass tubes provided by the manufacturer.

A practical drawback in applying gel permeation in the conventional mode is the time-consuming elution procedure caused by the inherent low flow-rates. The high compressibility of the gel does not permit flow-rates higher than can usually be achieved by hydrostatic flow. This problem has been overcome by implementation of the gel column in a continuous-flow system. The gel column is connected to a peristaltic pump so that the eluent is pumped in and sucked out at the same flow-rate. In this way the flow-rate can be increased with a minimum pressure build-up and gel permeation can be performed in an on-line mode with automated fraction collection. A further advantage of this technique is an improved reproducibility of selectivity and recovery of the clean-up method. The fraction containing the highest concentration of DEγE and the lowest concentration of interfering compounds as determined in the fractions composing the profile given in Fig. 2 is used for further pretreatment. The sample, with an initial volume of 1 ml, is diluted to about 6 ml during gel permeation, from which 3 ml (fraction I) is processed further and must therefore be concentrated again. This concentration step is performed on a reversed-phase XAD-2 column. The XAD-2 material is very suitable for this application, as described previously [9], because the capacity factor of DEγE is sufficiently high to allow preconcentration of volumes up to 10 ml without breakthrough. Direct analysis of fraction I, using an isocratic ion-pairing system that permits the separation of DEγE and its metabolites, and that is compatible with LIF detection, results in disappointing determination limits and unacceptable run times. Therefore, an additional clean-up by means of a gradient elution was applied. In this step the use of ion-pair formation must be avoided because this should introduce selectivity between the individual analytes. After 15 min, a heart-cut (fraction II with a volume of 0.5 ml) was made. Fig. 3a shows a chromatogram of the gradient run of a plasma sample, where the marked area indicates the heart-cut. Fig. 3b and c show the chromatogram of the heart-cut fractions containing 400 and 40 ng, respectively. Although this fraction seems to be fairly clean, it must be realized that in this stage neither an ion-pairing agent nor LIF detection was applied. Therefore, fraction II can now be analysed by an isocratic

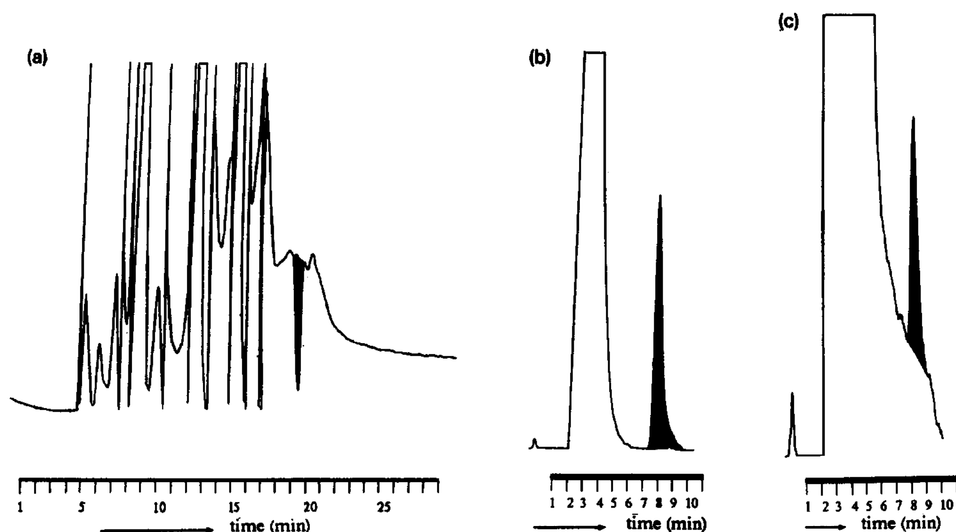

Fig. 3. Chromatogram of plasma sample after sample pretreatment with gel permeation, solid-phase concentration and gradient elution. For conditions, see text. (a) The black area indicated the location where the heart-cut was taken. Processed plasma volume, 1 ml. (b), (c) Chromatograms of heart-cut fractions (b + c); b and c are samples containing 400 and 40 ng of DEγE, respectively, added to 1 ml of plasma.

system, but first it must be diluted with water to reduce the modifier content. The diluted fraction with a volume of about 1 ml can be introduced on the isocratic system, either by concentrating the sample once again on an XAD-2 precolumn, or by injecting the sample with an injection loop of 1.5 ml applying on-column concentration.

With the ion-pairing chromatographic system DEγE can be separated from its metabolites βE 7–17 and βE 8–17 [19,20]. Because the sample pretreatment does not discriminate between DEγE and its metabolites, the recovery of these analytes is about the same. The recovery of the overall method was determined by assaying blank plasma samples that were spiked with known amounts of the compounds under investigation. The recovery for this group of peptides was about 50%. Although this recovery can be increased by selecting a larger volume after the gel permeation step, a smaller fraction was collected, because this step has been optimized with respect to the ratio between analytes and interfering compounds. Obviously, this lower recovery is due to the limited efficiencies achieved with permeation columns of the Sephadex type.

A conventional fluorescence detector was used to monitor the sample pretreatment, although of course a less sensitive method such as UV absorbance detection can also be used for this purpose. The LIF detection system has been used because pharmacokinetic profiles had to be followed down to the pg/ml range. If such low concentration levels are not involved, conventional fluorescence detection can also be used in the final separation and determination stage.

The analysis time for a single sample is about 80 min. However, a series of about 20 samples could be analysed in *ca.* 8 h. This higher throughput of samples is possible because except for the acidic deproteination and the last dilution step before

injection into the ion-pairing system, the system is automated and most steps can be operated simultaneously. Also, the two off-line steps can be integrated into the automated procedure, which can be realized by either robotics or continuous-flow techniques, although the former would more suitable for implementation.

Quantitative aspects

In order to generate calibration graphs, blank plasma samples were spiked with DEγE at concentration of 1, 5, 10, 50 and 100 ng/ml and analysed according to the procedure with LIF detection as described above. A typical correlation coefficient and equation of the curve were 0.997 and $y = 1.85\,(\pm 0.05)x + 2.54\,(\pm 2.48)$, respectively, in which the standard deviation of the slope and the intercept are given in parentheses. The fluorescence signal is expressed in arbitrary fluorescence units. The determination limit was 1 ng/ml plasma.

The accuracy of the method was determined by analysing a series of ten blank plasma samples spiked with 100 ng/ml of DEγE using conventional fluorescence detection. The accuracy was characterized as the relative difference between added and found concentrations and appeared to be -7%. This relatively high bias is certainly caused by the limited stability of the peptide. Degradation of DEγE was observed not only in biological matrices but also in buffered solutions (pH 2.4, 0.01 *M*, in demineralized water and in purified water sometimes unpredictable and unexplainable degradation was observed.

The day-to-day variation was determined on four different days, resulting in a relative standard deviation of 4.9% at a level of 100 ng/ml and 9.7% at 10 ng/ml.

Application to biological samples

The applicability of the method was demonstrated by assaying a number of plasma samples originating from a pharmacokinetic experiment. In Fig. 4a and b the chromatograms are shown of an extract of plasma from a healthy volunteer before and after intramuscular administration of 20 mg of DEγE. It can be seen that DEγE is

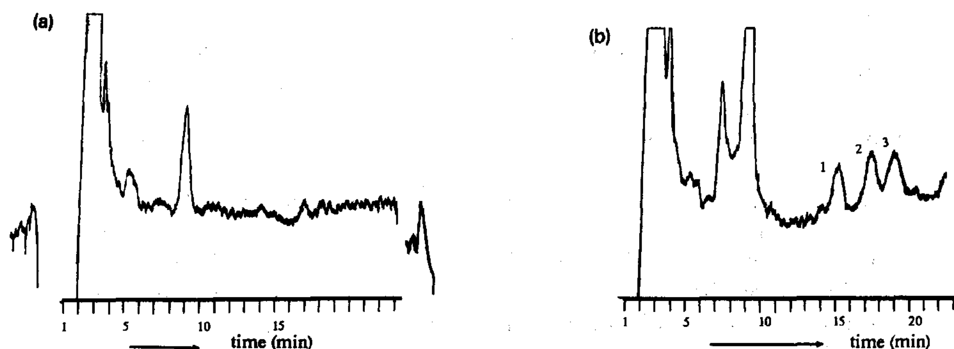

Fig. 4. (a) Analysis of human plasma sample before intramuscular injection of DEγE; 1 ml of plasma was analysed. (b) Analysis of human plasma sample taken 8 min after intramuscular injection of 20 mg of DEγE; 1 ml of plasma has been analysed. The peaks correspond to about (1) 4 ng/ml of DEγE, (2) 4 ng/ml of βE 7–17 and (3) 4 ng/ml of βE 8–17.

well separated from its main metabolites βE 7–17 and βE 8–17. The pretreated samples were measured with the LIF detection system. The metabolites of DEγE were determined from the calibration graphs for DEγE. This is possible only because the OPA derivatization takes place at the same amino function of peptides. The metabolic degradation took place at the C-terminus of the peptides [1]. Hence the fluorescence intensities of the OPA derivatives of DEγE and its metabolites can be considered to be equal.

CONCLUSIONS

The method presented is suitable for the determination of DEγE and its metabolites βE 7–17 and βE 8–17 at concentrations down to 1 ng/ml in complex biological matrices such as plasma. The method is more sensitive and selective than previously described methods such as on-line dialysis [9] and RIA [1]. The on-line dialysis method could not reach the same determination levels and the RIA method could not achieve the same selectivity.

A major problem common to all methods is the instability of DEγE in biological matrices at concentrations below 100 ng/ml. The risk of this instabiliy can be reduced to a minimum by extremely careful handling of the samples and peptide solutions. Peptide stock solutions were more stable in acidic buffers, but also in this environment degradation was sometimes observed.

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