Journal of Pharmaceutical & Biomedical Analysis
Vol. 8, No. 6, pp. 541–545, 1990
Printed in Great Britain

0731–7085/90 $3.00 + 0.00

Short Communication

An avidin–biotin based enzyme-linked immunosorbent assay for dynorphin A 1–13

GÜNTHER HOCHHAUS* and LASE HU

Department of Pharmaceutics, College of Pharmacy, University of Florida, Gainesville, FL 32610, USA

Keywords: *Enzyme immunoassay; dynorphin A 1–13; avidin–biotin system.*

Introduction

The avidin–biotin system has proven to be a versatile tool for the immunochemical detection of proteins [1]. We have previously described a sensitive competitive biotin–avidin based enzyme-linked immunoassay (ELISA) for the detection of β-endorphin in biological fluids [2]. In order to test whether this approach is also suitable for other opioid peptides with lower molecular weights we applied a similar system for the design of an ELISA for dynorphin A 1–13. Dynorphin A 1–13 amide is resistant to proteolytic cleavage [3] and can be easily biotinylated via the free amino groups of lysine. This derivative showed cross-reactivity to a commercially available dynorphin A 1–13 antiserum and is used in this study as an ELISA tracer. The assay is based on competition between dynorphin A 1–13 and biotinylated dynorphin A 1–13 amide for immobilized antibody binding sites, with subsequent detection of the antibody bound biotinylated species with enzyme labelled avidin.

Experimental

Materials

The following compounds were obtained from the sources indicated: CM-Affigel Blue from Biorad (Richmond, CA); ABC-KIT (consisting of avidin DH and biotinylated alkaline phosphatase) and alkaline phosphatase (AP)–avidin conjugate from Vector (Burlingame, CA); dynorphin A 1–13 amide, dynorphin A 1–13, thiorphan and dynorphin A 1–13 antiserum (Lot: 009025-2) from Peninsula (Belmont, CA); casein, diethanolamine, *p*-nitrophenyl phosphate, Tween 20, bovine serum albumin (BSA), biotinyl-aminocaproic acid *N*-hydroxysuccinimide ester (Biotin–XNHS), trifluoroacetic acid (freshly distilled) and 2′-(4′-hydroxyazobenzene)-benzoic acid (HABA) from Sigma; 96 well polyvinyl microtitration plates from Costar (Cambridge, MA); acetonitrile (HPLC grade) from Fisher; and captopril from Squibb (Princeton, NJ). All other chemicals were analytical grade.

Instrumentation

The liquid chromatograph consisted of a gradient solvent pump (LDC/Milton Roy, model CM4000), an injection system (Reodyne, model 7125), an analytical reversed-phase column (DeltaBond 300 Octyl, 5 μm, 15 cm × 4.6 mm, Keystone), a variable wavelength detector (LDC/Milton Roy, model 3100) and a strip chart recorder (Kipp & Zonen, model BD41). A microplate reader (Cambridge Technology series 700) was used in the ELISA procedure. A Kratos mass spectrometer (model MS 80, FAB mode) was used in the characterization of the biotinylated reaction products.

Buffers

The coating buffer was sodium carbonate–bicarbonate buffer (pH 9.6, 0.1 M); the blocking buffer was 1% casein, 10% monoethanolamine in sodium carbonate–bicarbonate buffer (pH 9.0, 1 M); 0.05% Tween 20 in phosphate-

*Author to whom correspondence should be addressed.

buffered saline (PBS) was used as the washing buffer; the incubation buffer was 0.5% BSA containing 30 μM bestatin, 0.3 μM thiorphan and 10 μM captopril; the substrate buffer consisted of 10% diethanolamine (pH 9.8) containing 3×10^{-4} M $MgCl_2$ and 2 mg ml^{-1} *p*-nitrophenyl phosphate.

Antiserum purification

Antiserum purification was performed by CM-Affigel Blue chromatography as recommended by the manufacturer. Briefly, the lyophilized antiserum (equivalent to 50 μl) was reconstituted in 100 μl of PBS and applied to a washed and settled gel (400 μl). The void volume obtained after washing with PBS was collected and stored after addition of 30% glycerol at −20°C. The protein content of the purified fraction was 0.5 mg ml^{-1} as determined by the method of Lowry [4].

Biotinylation of dynorphin A 1–13 amide

Forty microlitres of an aqueous solution of dynorphin A 1–13 amide (1 μg μl^{-1}) was mixed with 22 μg of biotin–XNHS (dissolved in 11 μl dimethyl sulphoxide) and 40 μl 0.1 M $NaHCO_3$. The mixture was incubated for 10 min at room temperature. Separation of the reaction products was achieved by reversed-phase liquid chromatography using a solvent gradient, a flow rate of 1 ml min^{-1} and UV detection at 210 nm. Solvent A was acetonitrile containing 0.1% trifluoroacetic acid. Solvent B was 0.1% trifluoroacetic acid in double-distilled water. A 30 min gradient from 20% A to 35% A was used. The peaks were collected, evaporated under a stream of nitrogen, dissolved in the incubation buffer and stored at 4°C. Under these conditions, the material was stable for at least 1 month as determined by chromatography of the stored material. Reaction products were analysed by fast atomic bombardment mass spectroscopy on a Kratos MS80.

Dissociation from avidin

One hundred microlitres of mono-biotinylated dynorphin A 1–13 amide (BX1-DYN, 10 μg ml^{-1} in PBS) was added to microtitration-plate wells and incubated overnight at 4°C. The wells were washed 4 times with PBS to remove unbound BX1-DYN and incubated with blocking buffer for 4 h at room temperature, washed again and then incubated with 100 μl of avidin–AP conjugate (5 μg/10 ml in PBS) for 45 min at room temperature. The dissociation process was initiated after different time intervals (0–4 h) by addition of an excess of biotin (10 μl of 0.1 M aqueous solution, pH 8.5) to the individual wells. Four hours after the first addition of biotin, all the wells were rinsed 5 times with washing buffer and incubated with 100 μl of substrate buffer for 20 min. The reaction was stopped by the addition of 50 μl 2 M NaOH and the absorbance was measured at 495 nm using a microplate reader. Control wells not coated with BX1-DYN, but treated otherwise in the same way, were used to determine non-specific binding, which was subtracted.

ELISA

In the optimized procedure, microtiter plates were incubated overnight at 4°C with 100 μl of a 1:500 dilution of purified antiserum prepared in the coating buffer. The coating solution was aspirated, the wells were rinsed four times with PBS and then blocked with 250 μl of blocking buffer. Binding studies were performed under either equilibrium or sequential saturation conditions. For the equilibrium method, BX1-DYN (0.5 nM) and various concentrations of dynorphin A 1–13 (prepared in incubation buffer) were mixed in a ration of 1:10. 100 μl of these solutions (final BX1-DYN concentration 0.25 nM) were transferred into the wells of the microtitration plate and incubated for 90 min at room temperature. For incubations under sequential saturation conditions, wells were preincubated with 100 μl of different concentrations of the standard solutions (prepared in incubation buffer) for 90 min at room temperature. After addition of 50 μl BX1-DYN (0.25 nM in the final incubation mixture) the mixture was incubated for an additional 45 min. For both equilibrium and sequential saturation experiments, wells were washed 4 times with washing buffer and 100 μl of ABC complex (prepared as recommended by the manufacturer in washing buffer containing 0.1% BSA) was added to the wells. After an additional 20 min, wells were washed 5 times with washing buffer and incubated at room temperature with 100 μl of substrate buffer. After signal development (generally 45 min) the reaction was stopped by addition of 50 μl of 2 N NaOH and the absorbance was measured at 405 nm using a microplate reader. All determinations were performed in quadruplicate. The limit of detection was defined as

the concentration of dynorphin A 1–13 for which the absorbance differed from the control incubations (absorbance of BX1-DYN in the absence of dynorphin A 1–13) by three times the standard deviation. Plasma samples used in this study were obtained from a female healthy volunteer and stored at −20°C. Aliquots were diluted with 10 vol of incubation buffer containing different concentrations of dynorphin A 1–13 (0–100 fmol ml^{-1}) and analysed as described for standard solutions using the sequential saturation method.

Results and Discussion

Hochhaus *et al.* [5] have recently described the *de novo* synthesis of [biocytin[13]] dynorphin A 1–13 amide as a potential probe for the κ-opioid receptor. This compound did not contain a spacer between the peptide backbone and biotin and showed a decreased receptor binding affinity in the presence of avidin. In order to minimize possible steric hindrance [6] that might affect sandwich formation between the antibody, biotinylated peptide and enzyme labelled avidin, biotin was introduced in this study via a C_6 spacer arm. Biotinyl-ε-aminocaproic acid *N*-hydroxysuccinimide ester reacts readily with free amino groups of peptides and was used for this purpose. The reaction yielded a mixture of biotinylated products as demonstrated by reversed-phase LC (Fig. 1). FAB analysis of peak 2 (BX1-DYN) revealed a molecular ion with *m/z* 1943. This was consistent with the theoretical molecular weight of the molecular ion (MH^+) of mono-biotinylated material. No molecular ion was observed for peak 3, (presumably bis-biotinylated material) because of instrumental limitations, which did not permit analysis of compounds with $m/z > 2000$. Isocratic liquid chromatography of peak 2 revealed two partially resolved peaks (data not shown) indicating that this material represented a mixture of mono-biotinylated compounds. Because of the limited resolution, further studies were performed however with the material obtained with the steep gradient system.

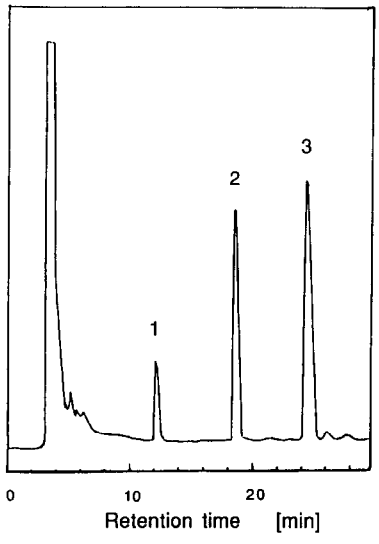

Figure 1
Liquid chromatographic analysis of biotinylated reaction products. Peak 1 represents unreacted dynorphin A 1–13 amide. Peaks 2 (BX1-DYN) and 3 represent presumably material with different biotinylation number.

Since it was only intended to use mono-biotinylated material in the ELISA procedure, it was further characterized by measuring the dissociation rate of the BX1-DYN–avidin complex. BX1-DYN was first adsorbed to the plastic surface of the microtitration plates and avidin–AP was bound to it. Dissociation was initiated by the addition of an excess of biotin. The dissociation half-life of the avidin–AP-BX1-DYN complex, as determined from a semi-logarithmic plot (8 h, data not shown), was comparable with previous results for the non-spacer derivative [biocytin[13]] dynorphin A 1–13 amide (10 h [5]). Greater stability has been reported for biotinylated insulin derivatives with a C_6 spacer arm compared with the corresponding derivatives prepared without spacers [6]. Differences in the experimental design, such as the use of alkaline phosphatase avidin conjugates instead of underivatized avidin, might be responsible for this observation. However, the experiment revealed that the avidin–AP-BX1-DYN complex is sufficiently stable for use as the tracer in an ELISA procedure.

A commercially available dynorphin A 1–13 antiserum was employed in the ELISA procedure after purification with Affigel Blue chromatography. This was done in order to ensure a high coating efficiency [7]. Coating of the plate wells with antiserum and the blocking of the remaining free plastic surface area was performed under conditions previously shown to be optimal for an ELISA for β-endorphin [2]. From the time course of binding at 20°C, incubation times were determined that ensured either equilibrium, in equilibrium binding studies (90 min), or a sufficient absorbance in the second step of the sequential saturation procedure without greatly disturbing the equi-

librium reached in the first step (45 min). Optimized antisera dilutions and tracer concentrations were selected from experiments in which the optical density for a series of tracer concentrations was determined as a function of antibody dilution in the presence or absence of excess of dynorphin. From the experiments the highest antibody dilutions were selected that provided a ratio of signal (absorbance in the absence of excess of dynorphin) to noise (absorbance in the presence of excess of dynorphin) of 5. These combinations were tested further in preliminary competitive binding assays to give the optimal assay conditions described in the Experimental section.

A competitive binding curve obtained under optimized equilibrium binding conditions (Fig. 2) revealed an IC_{50} value of 0.06 nM corresponding to 6 fmol per assay and a limit of detection of 1 fmol (3 × SD). A 6-fold increase in sensitivity was observed when sequential saturation conditions were employed, resulting in an IC_{50} value of 10 pM (1 fmol/assay) and a limit of detection of at least 0.3 fmol/assay (3 × SD). The IC_{50} value was consistent with that claimed by the manufacturer (Peninsula Laboratories) for a sequential saturation RIA procedure employing the same antiserum and ^{125}I-dynorphin A 1–13 as a tracer (IC_{50}:10 pM). The identical IC_{50} values imply that biotinylated and radioactive tracer label the same high affinity antibody binding sites and argue for the equivalence of both methods under optimized conditions. However, the IC_{50} value for the sequential saturation assay increased during storage of the purified antibody. After 3 months storage at −20°C, a 3-fold increase in the IC_{50} was observed. After 5 months, the antiserum exhibited an IC_{50} value of only 0.3 nM corresponding to 30 fmol/assay. Optimized storage conditions, such as lyophilization of the purified material or storage over liquid nitrogen, seems advisable when studies are planned over an extended period of time. However, the sensitivity of this assay, even after extended storage time, is comparable with other published radioimmunological procedures (30 fmol/assay [8]). The intra-assay RSD of the absorbances obtained for quadruplicates over the whole assay range was <6% in all experiments. The inter-assay RSD of four control samples (spiked with 1 fmol/assay, tracer conc.: 0.25 nM (n = 3), 0.5 nM (n = 1)) was 19% with a mean of 0.92 fmol/assay.

This system has been applied to the determination of dynorphin A 1–13 in biological material. Human plasma samples containing peptidase inhibitors were analysed with the 5 month antiserum and 0.7 pmol ml^{-1} dynorphin A 1–13 immunoreactive material was found. This compares well with literature values (0.48 pmol ml^{-1} [8]). Despite this good agreement, further tests were performed to test whether the presence of plasma might affect the results of the competitive binding experiments. The above characterized plasma (containing 0.7 pmol ml^{-1} of endogenous immunoreactive material), was spiked with known amounts of dynorphin A 1–13 (0–10 pmol ml^{-1}) and the concentrations were compared with plasma-free samples spiked with the same amounts (0–10 pmol ml^{-1}) of dynorphin. No systemic deviations in the results for plasma and plasma-free samples could be detected (Fig. 3). Hence, plasma did not seem to effect tracer or dy-

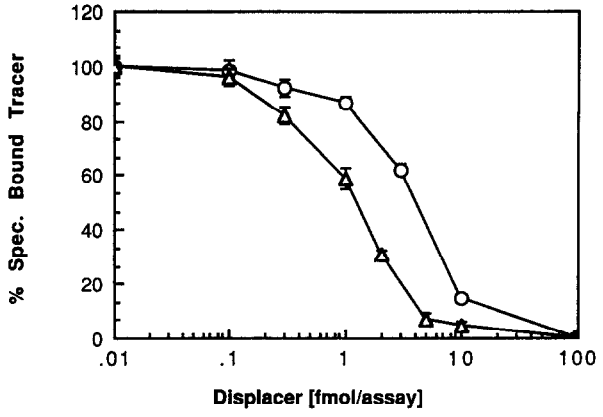

Figure 2
Standard curves for the assay of dynorphin A 1–13 employing equilibrium (○) and sequential saturation conditions (△).

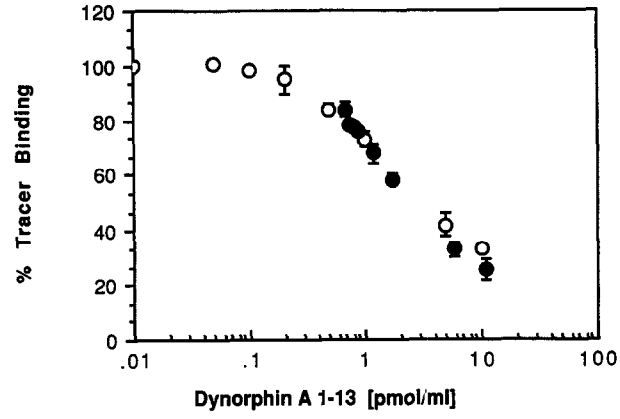

Figure 3
Influence of plasma on the binding of dynorphin A 1–13. Plasma containing 0.7 pM ml^{-1} dynorphin A 1–13 was spiked with different concentrations of dynorphin A 1–13 and analysed by the ELISA. Plasma free control samples spiked with the same amounts of dynorphin were assayed under identical conditions. Percent tracer binding observed for plasma (●) and control samples (○) are plotted against the actual dynorphin concentration.

norphin binding in a systemic matter. From this experiment the concentrations of spiked plasma samples were determined from a plasma free calibration curve. The observed concentrations were compared with the actual concentrations (endogenous level + amount added) by linear least-squares regression and the following equation was obtained: $y = 1.13x - 0.68$ (y and x: observed and actual concentrations, respectively, $n = 8$). The resulting RSD of 0.99 and the slope of 1.13 indicated that a reliable determination of dynorphin A 1–13 in human plasma could be performed under the given assay conditions without prior extraction. Hence, this method represents a fast and reliable alternative to established RIA procedures.

Acknowledgement — This study was supported by the University of Florida, College of Pharmacy Biomedical Research Support Grant.

References

[1] M. Wilchek and E.A. Bayer, *Analyt. Biochem.* **171**, 1–32 (1988).
[2] G. Hochhaus and W. Sadee, *Pharm. Res.* **5**, 232–235 (1988).
[3] F.M. Leslie and A. Goldstein, *Neuropeptides* **2**, 185–196 (1982).
[4] O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randell, *J. Biol. Chem.* **193**, 265 (1951).
[5] G. Hochhaus, A. Patthy, R. Schwieter, D.V. Santi and W. Sadee, *Pharm. Res.* **5**, 790–794 (1988).
[6] F.M. Finn, G. Titus and K. Hofman, *Biochemistry* **32**, 2554–2558 (1984).
[7] P. Tijssen, in *Laboratory Techniques in Biochemistry and Molecular Biology* (R.H. Burdon and P.H. van Knippenberg, Eds), Vol. 15, pp. 297–328. Elsevier, Amsterdam (1985).
[8] A. Valette, R. Desprat, J. Cros, G. Pontonnier, S. Belisle and S. Lemaire, *Neuropeptides* **7**, 145–151 (1986).

[Received for review 22 June 1989; revised version received 1 September 1989]