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Research Article

A Biotin–Avidin-Based Enzyme Immunoassay for β_h-Endorphin

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An avidin–biotin enzyme-linked immunosorbent assay (ELISA) is described for β_h-endorphin (β_h-EP). Microtiter plates coated with commercially available antibodies were used together with β_h-EP tracer derivatives that were biotinylated in positions 24, 28, and 29 via a C_6 spacer arm. Nonspecific binding of biotinylated derivatives to the microtiter plates was blocked with a mixture of 1% casein and 10% ethanolamine in 0.1 *M* $NaHCO_3$. A sequential saturation procedure using a high-affinity antiserum in combination with an avidin–alkaline phosphatase complex matched the sensitivity of reported radioimmunoassays (RIAs), with a detection limit of 0.5 fmol/assay. The intra- and interassay coefficients of variation were 5 and 12%, respectively. Results obtained by ELISA and RIA showed good correlations ($r = 0.95$). The β-EP concentration in extracted rat plasma after high-performance liquid chromatographic (HPLC) fractionation was determined by this method to be 1600 fmol/ml.

Key Words: β_h-endorphin; enzyme-linked immunosorbent assay (ELISA); biotin–avidin approach.

INTRODUCTION

Radioimmunoassays (RIAs)[4] currently provide the most sensitive method for measuring tissue levels of β_h-endorphin (β_h-EP) (1). We report here an ELISA procedure that is based on the biotin–avidin system (2,3) and equals the most sensitive ^{125}I-RIA reported (4,5).

We have previously synthesized several biotinylated β_h-EP derivatives as probes for the opiate receptor. β_h-EP derivative biotinylated at the carboxyl-terminal end of the molecule (Lys 24, 28, 29) via a C_6 spacer arm were able to cross-link the μ and δ opioid receptor with avidin. The same derivatives showed a high cross-activity to a β_h-EP antiserum (6) and were selected to develop an ELISA for β_h-EP. The assay is based on the competition of β_h-EP and biotinylated derivatives for antibody binding sites with subsequent enzymatic detection of antibody-bound biotinylated species with an avidin–enzyme complex. The assay is fast to perform, with a detection limit of less than 1 fmol β_h-EP per assay.

MATERIALS AND METHODS

Materials. ^{3}H-β_h-EP (sp act, 50 Ci/mmol) and unlabeled β_h-EP were kindly provided by Dr. Choh Hao Li (University of California, San Francisco). The following compounds were obtained from the indicated sources: CM-Affi-Gel Blue and MAP II^R binding buffer from Bio-Rad (Richmond, CA); avidin-DH, biotinylated alkaline phosphatase, biotinylated horseradish peroxidase, and avidin-D-alkaline phosphatase conjugate from Vector (Burlingame, CA); streptavidin and biotin-SP-alkaline phosphatase from Jackson Immunoresearch (Avondale, PA); human β_h-EP antiserum from Biogenex Laboratories (Dublin, CA; antiserum 1) or Peninsula (Belmont, CA; antiserum 2); 96-well polyvinyl plates from Costar (Cambridge, MA); lyophilized recombinant protein A from Repligen Corp. (Cambridge, MA); FMP-activated Fractogel (TSKAW-75-S) from Bio-Probe Int. (Tustin, CA); and biotinyl-ε-aminocaproic acid *N*-hydroxysuccinimide ester from Calbiochem (San Diego, CA). All other chemicals were of analytical grade.

Buffers. Coating buffer was 0.1 *M* sodium carbonate/biocarbonate buffer (pH 9.6); blocking buffer was 1% casein, 10% ethanolamine in 1 *M* sodium carbonate/bicarbonate buffer (pH 9.0); washing buffer was 0.05% Tween 20 in phosphate-buffered saline (PBS; pH 7.4); incubation buffer was 0.2% BSA and 0.01% bacitracin in washing buffer; and coupling buffer was 50 m*M* sodium bicarbonate, pH 8.5.

Antiserum Purification. Purification was achieved by either CM-Affi-Gel Blue chromatography (antiserum 2) or protein A chromatography (antiserum 1). In the case of CM-Affi-Gel Blue chromatography, 0.5 ml of settled gel was extensively washed with PBS. Lyophilized antiserum (dissolved in 100 μl PBS) was applied on the column, and the void volume obtained after the addition of PBS was collected and stored after the addition of glycerol (25%) at −20°C.

For protein A chromatography, recombinant protein A (2 mg) was dissolved in 10 ml of coupling buffer, and 200 mg of dry FMP-activated Fractogel was added. The suspension

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[4]Abbreviations used: β_h-EP, human β-endorphin; β-EP, β-endorphin; RIA, radioimmunoassay; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; OD, optical density; HPLC, high-performance liquid chromatography; MAPS^R, monoclonal antibody purification system; IRM, immunoreactive material; BSA, bovine serum albumin.

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was rotated overnight at 4°C. Excess of uncoupled ligand was washed off with 10 ml of coupling buffer and the remaining activated groups on the gel were deactivated by suspending the gel in 5 ml of 2% urea in 10 m*M* Tris buffer (pH 8.5) for 4 hr at 4°C. The gel was washed with 5 ml of each of the following solutions: 50 m*M* sodium citrate buffer (pH 4.0), 50 m*M* sodium citrate buffer (pH 4.0) containing 0.5 *M* NaCl, 10 m*M* Tris (pH 8.5); and 10 m*M* Tris (pH 8.5) containing 1.5 *M* NaSCN. The gel was washed with MAPS II binding buffer and stored at 4°C. One milliliter of serum was applied to 5 ml of gel, washed with 20 ml of MAPS II binding buffer, and eluted with 10 ml of 0.1 *M* sodium citrate buffer (pH 3.5). The gel was regenerated with 2 vol of 0.1 *M* NaOH, followed by 10 m*M* Tris buffer containing 1.5 *M* NaSCN.

Biotinylation of β_h*-EP.* Synthesis and purification of biotinylated derivatives were performed as described earlier (6). Briefly, 100 μg β_h-EP dissolved in 100 μl 0.1 *M* $NaHCO_3$ was mixed with 26.6 μg biotinyl-ε-aminocaproic acid *N*-hydroxysuccinimide ester (dissolved in 300 μl dimethyl sulfoxide). After 30 min of incubation at room temperature, the reaction was stopped by the addition of 50 μl 1 *M* HCl, and the reaction products were purified by reversed-phase HPLC (C_{18} μ-Bondapak column, Waters, Milford, MA) using a 40-min linear gradient of acetonitrile (A) and 0.1% trifluoracetic acid in water (B) (initial conditions, 28% A/72% B; final conditions, 34% A/66% B). The HPLC peak, corresponding to a mixture of derivative biotinylated in position 24, 28, or 29 (see Fig. 1), was concentrated under a stream of nitrogen until nearly dry, stored at 4°C, and used without further purification. The position of biotinylation was verified by HPLC analysis of tryptic digests and liquid secondary mass spectrometry (6). After evaporation of the HPLC eluent, derivatives were stable at 4°C for several months. Amounts of derivatives synthesized were calculated from the HPLC peak areas. In order to account for differences in the absorption coefficients between underivatized and derivatized β_h-EP, ^{3}H-β_h-EP diluted with unlabeled β_h-EP was biotinylated and the HPLC peak areas were normalized to the ^{3}H activity.

ELISA. Microtiter plates were incubated overnight at 4°C with 100 μl purified antiserum (diluted with coating buffer to give final protein concentrations of 20 μg/ml for antiserum 1 and 0.15 μg/ml for antiserum 2). The coating solution was aspirated, and the wells were rinsed four times with 250 μl washing buffer, blocked with 250 μl blocking buffer for 3 hr at room temperature, and again rinsed four times with washing buffer. Binding studies were performed under either equilibrium (antibody 1) or sequential saturation (antibodies 1 and 2) conditions. For the equilibrium method, 50 μl of biotinylated β_h-EP (concentration in final incubation mixture, 6 n*M* for antibody 1) and 50 μl of various concentrations of β_h-EP prepared in incubation buffer were added simultaneously and incubated for 1 hr at 37°C. This time period was sufficient to reach equilibrium. For incubations under sequential saturation conditions, wells were incubated with different concentrations of standard solutions (100 μl, prepared in incubation buffer) or actual samples (100 μl) at 37°C for 1 hr, followed by a 1-hr cooling period at 20°C. Degradation of peptides under the incubation conditions is unlikely, since EP has been proven

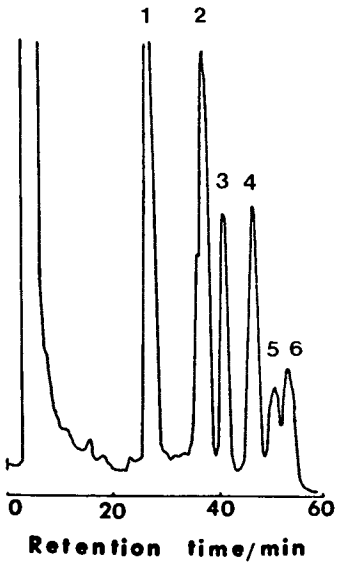


Fig. 1. HPLC chromatogram of biotinylated reaction products. Peak 2 corresponds to material monobiotinylated in positions Lys 24, 28, or 29 and was used in this study. Peak 1 represents unreacted β_h-EP, while peaks 3 and 4 correspond to material biotinylated at positions Lys 9 and Lys 19, respectively. Further derivatives with a higher biotinylation number (peaks 5 and 6) were also observed (see Ref. 6).

to be stable in plasma at 20 or 37°C (7,8). After the addition of biotinylated β_h-EP (10 μl; 6 n*M* for antiserum 1, 1 n*M* for antiserum 2), wells were further incubated for 1 hr at 20°C. Exactly after this time period, wells were washed four times with 250 μl washing buffer and incubated for 20 min at 20°C with the avidin-biotin alkaline phosphatase complex (100 μl; prepared in incubation buffer as recommended by the manufacturer). After washing five times, enzyme substrate (100 μl; 2 mg/ml *p*-nitrophenylphosphate in 10% diethanolamine-HCl, pH 9.8, containing 3×10^{-4} *M* $MgCl_2$) was added, and the mixtures were incubated at 20°C. The reaction was stopped by the addition of 50 μl *N* NaOH, and the optical density was measured in 1-ml cuvettes after the addition of 500 μl H_2O at 405 nm. Determinations were generally performed in triplicate. The limit of detection was defined as the concentration of β_h-EP for which the optical density differed from the control absorbance by three times the standard deviation. In preliminary experiments, horseradish peroxidase enzyme labels were used. In this case, an orthophenylenediamine solution [40 mg in citrate-phosphate buffer (50 m*M*, pH 5.0)], containing 0.002% H_2O_2, was used as substrate.

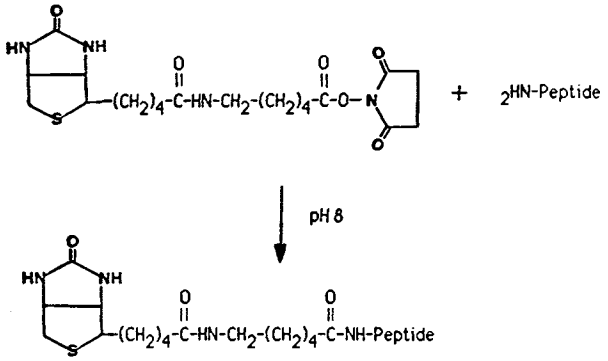
Extraction. Male Sprague–Dawley rats (200 g) were anesthetized with pentobarbital, blood was collected from the abdominal aorta; and bacitracin (0.01%) and sodium EDTA (1 mg/ml blood) were immediately added. Further sample handling was performed as described by Wiedemann and Teschemacher (4). Briefly, blood was centrifuged for 10

min at 1000*g* and 4°C. Plasma was carefully separated, acidified with 1 *N* HCl (100 μl/ml plasma), and centrifuged for 10 min at 18,000*g* to remove precipitates. The supernatant was extracted immediately with SEP Pak C18 cartridges (Waters) that were washed prior to use with 5 ml of methanol, 6 *M* urea, and 10 ml of water. After applying the plasma, cartridges were washed with 10 ml of water and 10 ml of 4% acetic acid. Retained peptides were eluted with 5 ml of a mixture of propanol and acetic acid (96/4, v/v.) The organic solvent was evaporated under a stream of nitrogen, and the extract was stored at −20°C. Reversed-phase HPLC separation (C18 u-Bondapak) of the extract was performed as described by Wiedemann and Teschemacher (4), using a 50-min linear gradient [initial solvent, acetonitrile/0.1% trifluoroacetic acid in water (15:85); final solvent, acetonitrile/0.1% trifluoroacetic acid (55:45, v/v); containing L-arginine, L-histidine, and L-tryptophane (50 μ*M* each)]. One-minute fractions were dried under a stream of nitrogen and assayed after redissolving in incubation buffer. Ninety to ninety-five percent of β-endorphin is recovered under these conditions (4).

RIA. Incubation conditions were adapted from the ELISA technique. Briefly, 200 μl purified antiserum 2 (final protein concentration, 0.15 μg/ml) was incubated with 200 μl sample or standard solution for 1 hr at 37°C. After a 1-hr cooling period at 20°C; ^{3}H-β_h-EP (15,000 dpm) was added and further incubated at 20°C for 1 hr. Bound and unbound ^{3}H-β_h-EP was separated by the charcoal method (6).

RESULTS AND DISCUSSION

The biotinylated β_h-EP tracer was found to bind with a high affinity to both β_h-EP antibodies after coating to the plate wells. Further, the bound tracer retained the ability to bind avidin. The C_6 spacer arm of the tracer was introduced to minimize potential steric constraints in the interaction of bound biotinyl tracer and avidin. A summary of the reaction is provided in Scheme I.



Scheme I. Synthesis of biotinyl-C_6 spacer peptide derivatives. Biotinyl-ε-aminocaproic acid *N*-hydroxysuccinimide ester reacts at basic pH with free amino groups of the peptide to form the corresponding amide.

In order to achieve a high sensitivity of the ELISA, it was necessary to optimize each step. The strong nonspecific binding of β_h-EP and related peptides to plastic necessitated blocking the remaining free surface area of the antibody-coated wells. Among the blocking agents tested, a mixture of 1% casein and 10% ethanolamine (pH 9.0) was the most effective in reducing the nonspecific binding of biotinylated β_h-EP, while 2% BSA or 1% casein alone was insufficient. The use of the blocking mixture resulted in optical densities (0.010–0.025) that were identical for control incubations (no addition of tracer) and incubations with biotinylated tracer (6 n*M*)) under nonspecific binding conditions (addition of 10^{-6} *M* β_h-EP). Hence, nonspecific binding of the biotinylated tracer was reduced to nondetectable levels.

We also tested different commercially available avidin–enzyme labels at concentrations recommended by the manufacturers. Such labels were either avidin–enzyme conjugates, with enzyme covalently bound to the avidin, or enzyme–avidin complexes that consist of complexes of biotinylated enzymes with avidin. Testing was performed in antiserum 1-coated microtiter plates after incubation with biotinylated β_h-EP (6 n*M*). An avidin–alkaline phosphatase conjugate (molar ratio of avidin to alkaline phosphatase, 1/1; 0.2 U/ml) yielded a measurable optical density only after overnight substrate incubation. An avidin–horseradish peroxidase complex and a horseradish peroxidase–streptavidin conjugate (7 μg/ml) were found to produce two times higher background levels than a complex consisting of streptavidin (4.1 μg/ml) and biotin-SP–alkaline phosphatase (3.4 μg/ml) or an avidin–biotinyl alkaline phosphatase complex, for which identical results were observed. The higher background for peroxidase preparations seems to be the result of nonenzymatic substrate oxidation. The nonspecific binding of streptavidin has been reported to be lower than for avidin (9); thus the identical results for avidin- and streptavidin–alkaline phosphatase complexes further demonstrate the optimal properties of the blocking buffer. For further studies, we used the avidin–alkaline phosphatase complex, at the ratio recommended by the manufacturer, as no improvement in the signal-to-noise ratio was observed upon variation of the avidin-to-enzyme ratio.

From the time course of tracer binding performed at different temperatures for both antisera used in this study, optimal incubation times were selected that allow either complete equilibrium during equilibrium binding studies (1 hr at 37°C) or sufficient optical density in step 2 of the sequential saturation assay without greatly disturbing the equilibrium reached in step 1 (1 hr at 20°C).

Finally, antibody dilution curves were performed for

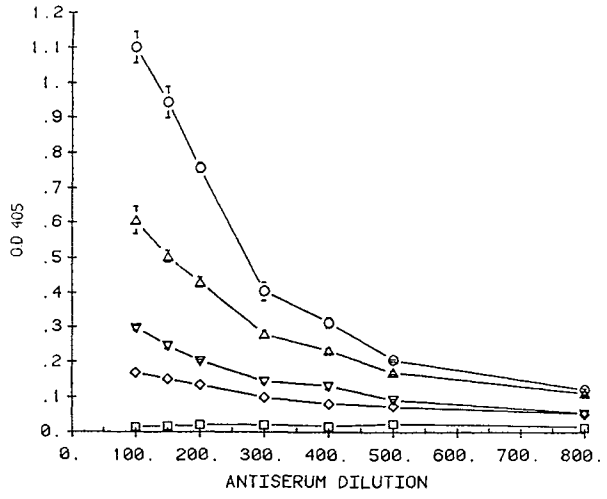


Fig. 2. Optical density as a function of antiserum dilution for different tracer concentrations. (○) 1 n*M*; (△) 0.5 n*M*; (▽) 0.2 n*M*; (◇) 0.1 n*M*; (□) nonspecific binding.

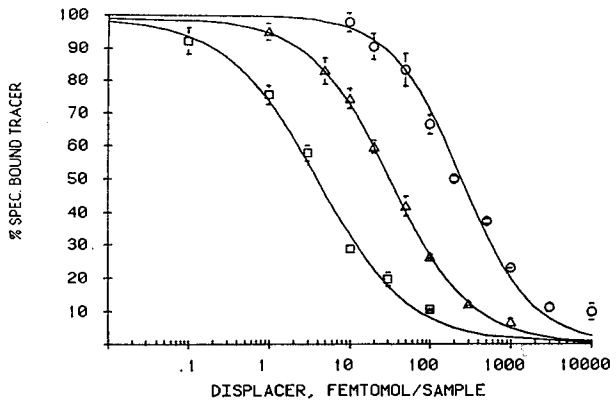


Fig. 3. Standard curves for the assay of β_h-EP using antiserum 1 under equilibrium (○) and sequential saturation conditions (△) and antiserum 2 under sequential saturation conditions (□).

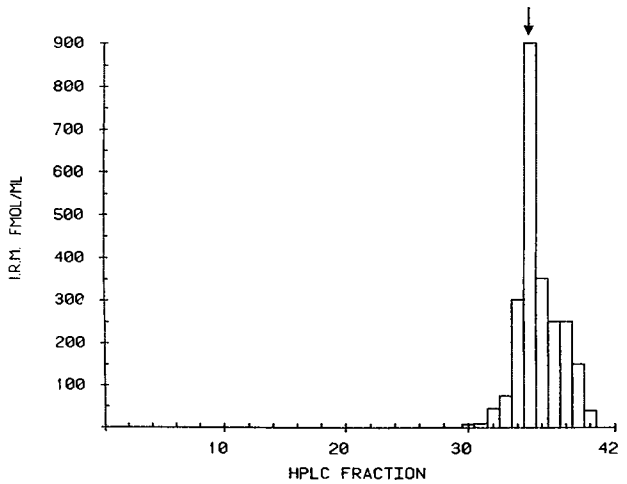


Fig. 4. Analysis of rat plasma extracts by HPLC–ELISA. Rat plasma was extracted and fractionated by reversed-phase HPLC. One-minute fractions were collected and assayed by ELISA. Arrows indicates elution position of β_h-EP. IRM, immunoreactive material.

both antisera using different tracer concentrations. From these experiments (shown for antibody 2 in Fig. 2), the highest antibody dilution for a given tracer concentration was selected that still provides a signal-to-noise ratio of 10:1.5. These combinations were further tested in preliminary competition experiments, resulting in the optimal assay conditions described under Materials and Methods.

Typical standard curves were obtained with both antisera under optimized conditions (Fig. 3). The change from equilibrium to sequential saturation conditions was accompanied by a seven fold increase in sensitivity, with an IC_{50} value of 32 fmol/assay and a limit of detection of 5 fmol/assay for antiserum 1. A further shift of the standard curve was observed when antibody 2 was applied under sequential saturation conditions (Fig. 3), resulting in an IC_{50} value of 5 fmol/assay and a limit of detection of 0.5 fmol/assay. The sensitivity observed with this high-affinity antiserum either matches or exceeds those of radioimmunoassays (4,5,10) and is five times higher than for the only other reported ELISA for β_h-EP (11).

The sequential saturation system was investigated in more detail. The intraassay variation of optical densities determined from quadruplicates over the whole assay range was less than 5%. Interassay variation for IC_{50} values (N = 5) and actual biological samples (N = 4) was 12 and 8%, respectively. In addition, results obtained by RIA and ELISA were compared. HPLC fractions of extracted rat plasma (see Materials and Methods) were measured by both methods. Data obtained with both assays for six samples containing 1.5–30 fmol/assay were analyzed by the least-squares method and the following equation was obtained: $y = 0.93x + 0.47$ (y and x: β_h-EP concentration as femtomoles obtained by RIA and ELISA, respectively). The resulting correlation coefficient of 0.95 indicated the equivalence of both methods.

We applied this system to the determination of β_h-EP in biological samples. Rat plasma was extracted, fractionated by reversed-phase HPLC, and analyzed by ELISA. The elution profile (Fig. 4) reveals that most of the immunoreactive activity coelutes together with authentic β_h-EP (fractions 32–36). However, we found additional immunoreactive material in eluate fractions 37–40, which do not correspond to β-EP. Similar material was detected by Wiedermann and Teschemacher in human plasma (4). Because of the high cross-reactivity of antiserum 2 for rat β-endorphin (92%; data of the manufacturer), the concentration of rat β-endorphin immunoreactive material could be determined from these data, assuming an identical extraction recovery for human and rat β-endorphin. Recognizing this limitation, the resulting β-EP concentration (1600 fmol/ml plasma) was calculated to be higher than the basal β-EP levels reported in the literature [100–430 fmol/ml (10–13)]. This may be explained by different conditions during the blood sampling procedure.

The results clearly demonstrate that the biotin–avidin-based ELISA is reproducible and as sensitive as reported RIAs without the need to use radioactive tracers.

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REFERENCES

1. J.-S. Hong, K. Yoshikawa, and R. W. Hendren. *Methods Enzymol.*103:547–564 (1983).
2. J. Korpela. *Med. Biol.* 62:5–26 (1984).
3. E. A. Bayer and M. Wilchek. *Methods Biochem. Anal.* 26:1–45 (1980).
4. K. Wiedemann and H. Teschemacher. *Pharm. Res.* 3:142–149 (1986).
5. C. Cahill and H. Akil. *Life Sci.* 31:1871–1873 (1982).
6. G. Hochhaus, B. W. Gibson, and W. Sadee. *J. Biol. Chem.* 263:92–97 (1988).
7. H. Sato, Y. Sugiyama, Y. Sawada, T. Iga, and M. Hanano. *Life Sci.* 35:1051–1059 (1984).
8. J. Rossier, A. Bajon, T. M. Vargo, N. Ling, R. Guilleman, and F. Bloom. *Life Sci.* 21:847–852 (1977).
9. M.-T. Haeuptl, M. L. Aubert, J. Djiane, and J.-P. Kraehenbuhl. *J. Biol. Chem.* 258:305–314 (1983).
10. V. Hoellt, R. Przewlocki, and A. Herz. *Naunyn-Schmiedeberg Arch. Pharmacol.* 303:171–174 (1978).
11. J. K. Sarma, S. R. Hoffmann, and R. A. Houghten. *Life Sci.* 38:1723–173 (1986).
12. R. Guillemin, N. Ling, and T. Vargo. *Biochem. Biophys. Res. Commun.* 77:361–366 (1977).
13. J. Rossier, E. D. French, C. Rivier, N. Ling, R. Guillemin, and F. E. Bloom. *Nature* 170:618–620 (1977).