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Improved clean-up method for the enkephalins in plasma using immunoaffinity chromatography

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Abstract

A rapid and selective method of sample clean-up using immunoaffinity chromatography (IAC) was developed to isolate enkephalins from plasma. The enkephalin antibodies were produced utilizing novel protein carriers. Two antibodies, LE4H8 and 33FC6, were selected because of their moderate binding affinity and different epitopes. Enkephalin-spiked plasma was loaded onto the immunoaffinity column and eluted with acidic pH buffer. The eluate was derivatized with naphthalene-2,3-dicarboxaldehyde in the presence of cyanide (NDA-CN), and the enkephalins were separated using reversed-phase liquid chromatography (RPLC). IAC sample clean-up of enkephalin-spiked plasma was compared to the existing solid-phase extraction method. The limit of detection for IAC was 30 pmol. The recovery of the enkephalins from plasma was 90% with a variance ranging from 2 to 9%. The immunoaffinity column was used for approximately 70 samples without any deterioration in performance.

1. Introduction

Leucine- and methionine-enkephalin (leu-enk and met-enk, respectively) belong to the family of neuroactive peptides known as the opioid peptides. Since their discovery [1,2], various methods have been employed in the analysis of the enkephalins. These methods include immunodetection [3,4] and liquid chromatography [5]. Perhaps the most important step in an analysis is pretreatment of the sample. A highly selective sample pretreatment method is crucial to the accurate determination of opioid peptides, such as leu-enk and met-enk. The enkephalins are present at only trace levels in most biological matrices. The concentration of enkephalins in tissue ranges from low pmol/g tissue to nmol/g tissue, and the levels in plasma and cerebrospinal fluid are present only in the low pmol/ml range. Great variability in enkephalin levels may arise because existing sample clean-up methods do not provide rapid and efficient removal or inactivation of proteolytic enzymes. The presence of enzymes can either lead to reduced peptide concentrations by degradation of the peptide itself and/or to increased concentrations by degradation of precursor peptides.

The existing sample pretreatment methods for the enkephalins are long, tedious, and can lead to poor, irreproducible recovery. The multi-step procedure begins with the acid precipitation of proteins, followed by neutralization, and further purification by liquid–liquid extraction [6–8] or multi-step solid-phase extraction [9–11].

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An alternative sample pretreatment method is immunoaffinity chromatography. Immunoaffinity chromatography (IAC) takes advantage of the specificity and affinity of the interaction between an antigen and its antibody. The strength and selectivity of antibody–antigen interactions promotes a high degree of purification. Purification factors range from 1000 to 10 000-fold [12]. Antigen yield typically ranges from 40 to 70% and can be as high as 90% [12]. Immunoaffinity chromatography is a one-step clean-up method, which allows for simple and fast pretreatment of biological matrices, such as plasma and serum, urine, or tissue and plant homogenates.

Enhanced immunization methods were employed in the production of antibodies to the enkephalins. These methods include the use of novel carrier proteins. Antibody LE4H8 was produced using antibodies that target the immune system as carrier proteins. The use of T- and B-cell antibodies as carrier proteins to enhance the immunogenicity of the enkephalins has been described in Hendrickson *et al.* [13]. Antibody 33FC6 was produced using the protein component of tuberculin (purified protein derivative or PPD) as the carrier protein. PPD is an interesting example of what could be called a T-cell hapten [14]. That is, PPD is recognized by and stimulates T-cells, but it does not elicit antibody production. PPD is prepared from the culture supernatants of *Mycobacterium tuberculosis*. It is not a pure protein, but the molecular mass of its main component is 10 000. It is advantageous to use PPD as the protein carrier, because it can be coupled to leu-enk to prepare peptide antibodies while generating virtually no antibody response against itself.

The purpose of this research was to develop a rapid, high yield, and selective clean-up method for leu- and met-enk in plasma using immunoaffinity chromatography. The simplicity, yield, and degree of purification of immunoaffinity pretreatment and solid-phase extraction (SFE) are compared. The pretreated samples were derivatized with naphthalene-2,3-dicarboxaldehyde in the presence of cyanide (NDA-CN) [15,16] and then analyzed using liquid chromatography. The NDA-CN is a fluorogenic reagent for primary amines. The introduction of a fluorescent tag on the enkephalins allows lower detection limits. The possibility of using the prepared immunoaffinity column in the analysis of other members of the opioid peptide family is also briefly discussed.

2. Experimental

2.1. Materials

Synthetic leu-enk (tyr-gly-gly-phe-leu), met-enk (tyr-gly-gly-phe-met), various enkephalin-related peptides, aprotinin, and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO, USA) and used as received. Freund's complete and incomplete adjuvant (CFA and IFA) were purchased from Gibco Laboratories (Grand Island, NY, USA). Pooled human plasma was purchased from the Community Blood Bank (Kansas City, KS, USA). Taurine was purchased from Aldrich (Milwaukee, WI, USA). NDA was obtained from Bioanalytical Systems (West Lafayette, IN, USA). Reactigel HW-65 was purchased from Pierce (Rockford, IL, USA). Acetonitrile and trifluoracetic acid (TFA) were HPLC-grade and were purchased from Fisher (St. Louis, MO, USA). Nanopure pure water (Barnstead Nanopure II, Sybron/Barnstead, Boston, MA, USA) was used throughout, and the other chemicals employed were of the highest purity available.

2.2. Production of antibodies

The production of antibody LE4H8 is described in ref. 13. Monoclonal antibody 33FC6 was produced in this laboratory. The immunogen was prepared by coupling leu-enk to purified protein derivative of tuberculin (provided by Lederle Laboratories, Pearl River, NY, USA), using the glutaraldehyde coupling method [17]. The immunogen was dissolved in phosphate buffered saline to 1 mg/ml. Bacillus Calmette–Guerin positive $(Balb/c \times C57BL/6)F_1$ mice were immunized intravenously with 30 μg of the immunogen. Immunization was repeated six

times every two weeks. The immunogen was emulsified in an equal volume of CFA and the mice were immunized intraperitoneal with 10 μg. Immunizations with 10 μg, in saline, were repeated every two weeks for 10 weeks. Two weeks before the cell fusion, the mice were immunized with 30 μg of the immunogen emulsified in an equal amount of IFA. The final boost was 40 μg in saline. The spleen cells were fused with P3x63-Ag8.653 myeloma cells according to the procedure described in ref. 18. The positive colonies were cloned by limited dilution, and expanded in the peritoneal cavity of athymic nude mice.

The methods used in the characterization (class, affinity constant, and cross reactivity) of LE4H8 and 33FC6 have been previously outlined [13].

2.3. Preparation of the immunoaffinity column

The antibodies were coupled to Reactigel according to the manufacturer's directions. 33FC6 and LE4H8 were coimmobilized (1 mg/ml each) to 1 ml of gel. The gel was tumbled end-over-end at 4°C for 24 h. The gel was filtered through a fritted glass disk funnel, and remaining active sites were deactivated with 2.0 *M* Tris buffer, pH 8.0. The amount of unreacted antibody was determined using the BCA protein assay (Pierce). The non-specific or weakly bound antibody was removed by washing with 0.1 *M* phosphate buffer (PB), pH 2.2, and 0.1 *M* PB, pH 7.4. The gel was packed into a stainless steel column with a volume of 680 μl (Upchurch Scientific, Oak Harbor, WA, USA). Bovine serum albumin (5 mg/ml) was injected and eluted multiple times to block non-specific adsorption sites on the gel. The immunosorbent was stored at 4°C in 0.1 *M* PB, pH 7.4 containing 0.1% NaN_3. The immunoaffinity column was stored at 4°C when not in use.

2.4. Solid-phase extraction (SFE)

Pooled human plasma was incubated at 4°C overnight to insure complete degradation of endogenous enkephalins. The samples were processed according to the procedure described in ref. 9 with the following modifications. The plasma (950 μl) was spiked with equal amounts of leu-enk and met-enk (32.0 nmol, 1.3 nmol, 51.2 pmol, and 0 mol each, in 50 μl nanopure water) and incubated 3 to 5 min before deproteinization. The final residue was reconstituted in 560 μl 0.1 *M* PB, pH 6.8, and was derivatized with NDA-CN. The derivatized enkephalins were analyzed by RPLC.

2.5. Immunoaffinity chromatography

The chromatographic equipment was obtained from Shimadzu Instruments (Kyoto, Japan). The system consisted of two Model LC-6A pumps, a Model FCV-2AH high pressure valve, a Model SIL-6A autoinjector, and a Model SPD-2AM UV detector. The data were recorded using the Model C-R4A Chromatopac data system. The system was controlled by a Model SCL-6A controller.

Pooled human plasma was incubated at 4°C overnight to insure complete degradation of endogenous enkephalins. The plasma (160 μl) was spiked with equal amounts of leu-enk and met-enk (12.8 nmol, 2.6 nmol, 510.0 pmol, 100.0 pmol, and 0 mol each, in 40 μl nanopure water). Aprotinin (10 000 KIU/10 ml plasma)[a] was also added to inhibit enzymatic degradation. The plasma was incubated 3 to 5 min and injected (50 μl) into a flowing stream (0.1 *M* PB, pH 7.4) that passed through the immunoaffinity column. The column was washed for 10 min with 0.1 *M* PB, pH 7.4. The enkephalins were eluted by passing 0.1 *M* PB, pH 2.2, through the column for 5 min. The column was reequilibrated with 0.1 *M* PB, pH 7.4, and was ready for another sample. The flow-rate used throughout the procedure was 0.2 ml/min. The bound fraction (elution volume = 280 μl) was collected into 280 μl of 0.2 *M* PB, pH 8.0, (the final pH was *ca.* 6.8) and 50 μl of 200 m*M* ascorbic acid. The

[a] KIU = Kallikrein inactivating unit; 1 KIU has the ability to inhibit 2 kallikrein units by 50% under optimum conditions.

peptides were derivatized with NDA-CN. The standards were also derivatized in this 1:1 mixture of 0.1 *M* PB, pH 2.2, and 0.2 *M* PB, pH 8.0. The derivatized enkephalins were analyzed by RPLC.

2.6. Derivatization procedure

Leu-enk and met-enk (standards were dissolved in nanopure water) were converted to their 1-cyanobenz[*f*]isoindole (CBI) derivatives according to ref. 10, except that the volume of taurine (200 m*M*) added to quench the reaction was 20 not 50 μl.

2.7. Liquid chromatography

The chromatographic equipment was the same as that described above, except that a Model RF-535 fluorimetric detector (xenon lamp, λ_{em} = 520 nm, λ_{ex} = 490 nm) was employed as detector. A Nucleosil C_{18} column (5 μm, 150 × 4.6 mm I.D.) was obtained from Alltech (Deerfield, IL, USA).

Separation was carried out at room temperature, and the mobile phase used was 40% (v/v) acetonitrile in 26 m*M* TFA (pH 3.5) with a flow-rate of 1.0 ml/min.

3. Results

3.1. Characterization of antibodies

Both antibodies, LE4H8 and 33FC6, are IgG. The apparent affinity constants (k_{app}) of LE4H8 and 33FC6 are $2.0 \times 10^7\ M^{-1}$ and $1.7 \times 10^7\ M^{-1}$, respectively as determined by serial dilution [13]. The relative cross-reactivities of each antibody towards known synthetic peptides are shown in Table 1. The cross-reactivities displayed by each antibody aid in the determination of their respective binding site or epitope. The panel of peptides was selected because of the differing sequences and conformations. It can be seen that LE4H8 binds to the amino terminal of the enkephalin sequence, and 33FC6 binds to the carboxyl terminal of the enkephalin sequence. When modifications are made to the amino terminal, the binding of LE4H8 is drastically reduced. The same is true for the binding of 33FC6 when changes are made on the carboxyl terminal. It is surprising that when the tyr residue was deleted, 33FC6 binding was substantially lowered. It is also surprising that when an arg residue or NH_2 group was added to the carboxyl terminal, the binding of LE4H8 was enhanced. These results indicate that the anti-

Table 1
Relative cross reactivities of antibodies LE4H8 and 33FC6

Peptide	Binding (%)	
	LE4H8	33FC6
Leu-enk (tyr-gly-gly-phe-leu)	100	100
Met-enk (tyr-gly-gly-phe-met)	32	59
3,5-Dibromo-tyr-gly-gly-phe-leu	18	95
Tyr-D-ala-gly-gly-phe-leu	42	85
Gly-gly-phe-leu	18	33
Tyr-gly-gly-phe-leu-arg	245	9
Tyr-gly-gly-phe-leu-NH_2	135	7
Tyr-D-ala-gly-phe-D-leu	13	6
β-Endorphin[a](tyr-gly-gly-phe-met-thr-ser-glu-lys-ser-gln-thr-pro-leu-val-thr-leu-phe-lys-asn-ala-ile-val-lys-asn-ala-his-lys-lys-gly-gln)	+[b]	−[c]

[a] β-Endorphin was adsorbed on the plate at 5 μg/ml in the absence of BSA. The percent binding cannot be compared to the other peptides.
[b] Indicates binding.
[c] Indicates no binding.

bodies bind to a non-linear conformation of the enkephalins. The antibodies must bind the enkephalins in a folded conformation where the amino and carboxy terminals are brought into close contact. The enkephalins are thought to exist in both a linear and β-bend conformation. The results here clearly indicate that antibodies LE4H8 and 33FC6 bind to the β-bend conformation. It is also evident that both antibodies display extensive cross reactivity to other related opioid peptides. Cross reactivity and epitope determinations by competitive ELISAs are described elsewhere [13].

3.2. Recovery of leu-enk and met-enk

The recovery of leu-enk and met-enk using SFE was never more than 50%. Recoveries of 80% have been reported [9,10].

The recovery from the immunoaffinity column is shown in Table 2. The recovery of leu-enk is approximately 90% at the pmol level. It can be seen that the recovery is quite reproducible. A side-product of the NDA-CN reaction coelutes with met-enk, therefore selective quantitation of met-enk at low levels is impossible. It is crucial to add aprotinin to the enkephalin-spiked plasma, because the enkephalins are so susceptible to enzymatic degradation. It has been reported that the half-life of the enkephalins in plasma is as short as a few seconds [19]. If aprotinin was not added, the spiked-enkephalins degraded before the plasma could be loaded onto the immunoaffinity column. The recovery of the enkephalins, in the absence of aprotinin, was never more than 50%.

3.3. Binding capacity and stability of the immunoaffinity column

The antibodies were coupled to Reactigel with approximately 100% efficiency. The capacity of each immunoaffinity column was determined by multiple injections (10 μl) of leu-enk and met-enk (3.2×10^{-9} *M*, in 0.1 *M* PB, pH 7.4) under flow conditions. The capacity of the column was studied after the column had been cycled *ca.* five times. There is always an initial decrease in capacity of immunoaffinity columns during the first few runs. This loss of capacity is due to the leaching of weakly bound antibody or to irreversible denaturation [20]. The true capacity cannot be found until this decrease reaches a plateau. The capacity of the immunoaffinity column was 3.2×10^{-9} mol enkephalin. The percentage of active antibody (ratio of active antibody to total antibody bound) was 38%. This percentage is typical for immobilization through the amine groups of the antibody.

It can be seen in Table 2 that there is a sharp decrease in recovery when the total amount of enkephalin loaded onto the column is 3.0×10^{-10} mol or more. This decrease in recovery indicates that the capacity of the immunoaffinity column is being approached. In order to achieve reproduc-

Table 2
Relative recoveries of leu-enk and met-enk spiked plasma using IAC clean-up

Enkephalin loaded (mol)	Recovery (mean ± S.D., $n = 4$)(%)		R.S.D. (%)	
	Leu-enk	Met-enk	Leu-enk	Met-enk
$3.0 \cdot 10^{-11}$	98 ± 2	–[a]	2	–
$6.0 \cdot 10^{-11}$	90 ± 4	–	4	–
$1.2 \cdot 10^{-10}$	83 ± 5	103 ± 5	6	5
$3.0 \cdot 10^{-10}$	67 ± 5	70 ± 6	7	9
$6.4 \cdot 10^{-10}$	54 ± 3	53 ± 3	5	6
$3.2 \cdot 10^{-9}$	50 ± 2	43 ± 4	4	9

[a]Could not be determined due to interference peak caused by a side product of the NDA-CN reaction.

ible high recoveries, the amount of sample loaded should be 100 times lower than the capacity of the column. The capacity of the column is adequate for the levels of enkephalin found in plasma and most tissue samples.

The immunoaffinity column was used approximately 70 times, and no change in capacity or recovery was found.

3.4. Chromatographic analysis of leu-enk and met-enk

Figs. 1 and 2 show typical RPLC chromatograms of blank and enkephalin-spiked plasma using SFE and IAC clean-up. The large void peak (due to taurine) is considerably larger in the IAC samples. The addition of taurine is necessary due to the instability of the fluorescent

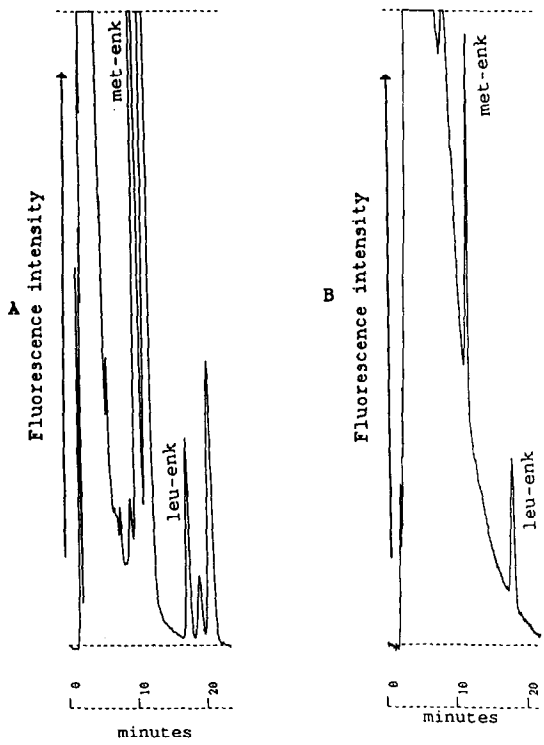

Fig. 1. RPLC chromatograms, with fluorescence detection ($\lambda_{em} = 520$ nm, $\lambda_{ex} = 490$ nm), of a plasma blank . The clean-up was (A) SFE and (B) IAC. Stationary phase: Nucleosil C_{18}; mobile phase: 40% (v/v) acetonitrile in 26 m*M* TFA, pH 3.5; flow-rate: 1 ml/min; temperature: ambient.

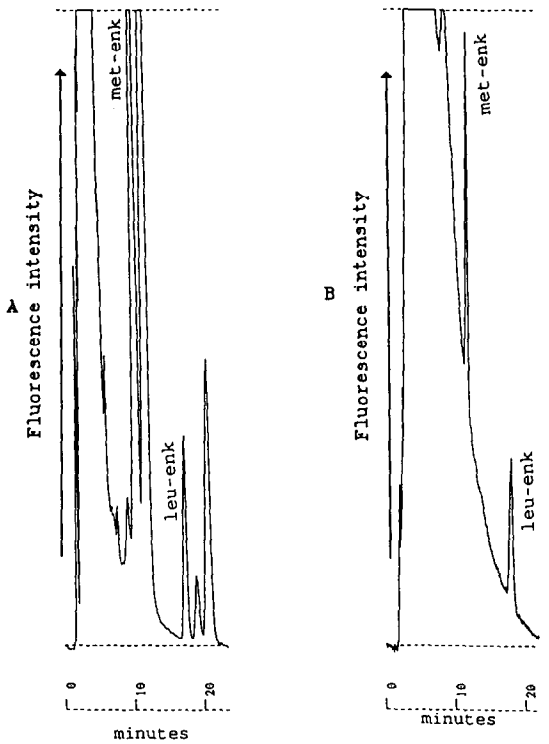

Fig. 2. RPLC chromatograms, with fluorescence detection ($\lambda_{em} = 520$ nm, $\lambda_{ex} = 490$ nm), of plasma spiked with (A) 1.3×10^{-9} mol leu-enk and met-enk using SFE clean-up, and (B) 3.0×10^{-10} mol leu-enk and met-enk using IAC clean-up. Stationary phase: Nucleosil C_{18}; mobile phase: 40% (v/v) acetonitrile in 26 m*M* TFA, pH 3.5; flow-rate: 1 ml/min; temperature: ambient.

derivatives in the presence of excess NDA-CN. The blank and enkephalin-spiked plasma samples were analyzed without the addition of taurine in order to determine if interfering peaks were hidden under the large void peak. When the plasma was analyzed in the absence of taurine, no interfering peaks were found in the RPLC chromatograms. The limits of detection of leu-enk using SFE and IAC clean-up are 51 pmol and 30 pmol, respectively. Selective quantitation of met-enk is impossible at low levels due to interference from the NDA-CN reaction. The detection of the enkephalins was limited by the chromatography, not by the recovery or capacity of the immunoaffinity column. These values are comparable to those found previously using a similar chromatographic system [9,21].

4. Discussion

Compared to solid-phase extraction, the use of immunoaffinity chromatography greatly reduces the time and number of steps in the clean-up procedure. The enkephalins are isolated in less than 20 min. Immunoaffinity chromatography is easily automated, so the manual labor is virtually eliminated. The enkephalins are isolated and concentrated all in one step. The volume of sample loaded onto the immunoaffinity column is only limited by the capacity of the column. In the case of enkephalins, 10 to 100 ml of plasma can be injected onto the column without overloading. The pre-concentration, then is quite impressive. The immunoaffinity column was used for approximately 70 samples without loss in performance. It is true that monoclonal antibodies can be expensive, but immobilized antibodies are cost-effective when compared to the high price of single-use solid-phase extraction cartridges.

The most significant advantage of immunoaffinity chromatography can be seen in Figs. 1 and 2. The high degree of purification that immunoaffinity chromatography provides is obvious. Fig. 1 shows a plasma blank that has been subjected to SFE and IAC clean-up. It can be seen that there are several peaks present in the chromatogram of the plasma blank that underwent SFE clean-up. These peaks are due to species that were extracted from the plasma blank on the basis of their hydrophobicity. SFE cannot differentiate between the enkephalins and other species with similar hydrophobicity. The only peak that is present in the chromatogram of the plasma blank that underwent IAC clean-up is the large void peak of taurine derivatized with the excess NDA-CN. The taurine peak is large because there are no other derivatizable species present in the sample, whereas SFE left several derivatizable species in the plasma blank. These interferences are again seen in the enkephalin-spiked plasma with SFE clean-up shown in Fig. 2. It is clear that extraction on the basis of hydrophobicity is not selective. Immunoaffinity chromatography has the ability to selectively isolate the enkephalins from a spiked plasma sample. The chromatogram of spiked plasma with IAC clean-up shows only the presence of leu-enk and met-enk. The present results demonstrate that immunoaffinity chromatography provides a fast, simple, and efficient means of sample pretreatment. The recovery and limit of detection are comparable to solid-phase extraction and it is much faster and easier. The amount of purification that immunoaffinity chromatography provides far exceeds that of solid-phase extraction. The limit of detection can be improved by two orders of magnitude when multi-dimensional chromatography is used [10]. Multi-dimensional chromatography would also allow for improved quantitation of met-enk by improved separation of met-enk from the interference of the NDA-CN reaction.

Many of the peptides in the opioid family contain either the sequence of leu-enk or met-enk, therefore other opioid peptides and metabolites could also be purified by taking advantage of the defined cross reactivities of LE4H8 and 33FC6. The combination of antibodies is most effective because the antibodies recognize opposite ends of the enkephalin sequence, thereby capturing opioid peptides, which contain amino acids from either the amine or carboxy end of the enkephalin sequence. The development of derivatization agents, such as NDA-CN, has provided significant improvement in the detection of amino acids and small peptides at low levels. These derivatization agents are quite applicable to the smaller peptides in the opioid family, but there are very few methods that enable detection of the larger opioid peptides at the trace levels that are present in biological matrices. The analysis of the entire family of opioid peptides, following immunoaffinity chromatography clean-up, must wait upon further developments in detection methods.

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