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# High-performance liquid chromatographic resolution of synthetic opiate and "anti-opiate" peptides from human plasma

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**Abstract**

An assay system using reversed-phase high-performance liquid chromatographic (HPLC) resolution of synthetic anti-opioid peptides (AOPs) and opioid peptides (OPs) was developed. Samples were diluted with trifluoroacetic acid, loaded onto Sep-Pak $C_{18}$ cartridges, eluted, dried, and redissolved in ethanol–acetic acid–water. Retention-time consistency was established, and high levels of synthetic AOP and OP recovery, generally higher than 80%, were achieved. In a single HPLC run synthetic enkephalins, dynorphins, and $\beta$-endorphins were separated even when extracted from human plasma using a volatile mobile phase which yielded fractions totally compatible with quantitation by radioimmunoassay. Combining the resolution of HPLC with the sensitivity of radioimmunoassay (RIA) may facilitate simultaneous measurement of numerous neuropeptides in body fluids such as plasma and cerebrospinal fluid.

## 1. Introduction

According to the anti-opioid model of tolerance and dependence [1], the central nervous system secretes peptides that attenuate the effects of endogenous opioids. Some of these putative anti-opioid peptides (AOPs) and endogenous opioids (OPs), such as [Met$^5$]-enkephalin and dynorphin A 1–17, might modulate opioid tolerance and dependence by competing with morphine for the opioid binding site. The role of AOPs and OPs in opioid-induced tolerance is the subject of considerable research. Nyberg et al. [2] recently reviewed opioid peptides, including an emphasis on correlations with clinical disorders.

We sought to establish a method to quantitate plasma and cerebrospinal fluid AOP and OP concentrations, with the eventual objective to determine if these concentrations are altered by opioid abuse. In addition, it was felt that the ability to quantitate multiple AOPs and OPs from the same sample would provide much better understanding of the effect of drugs of abuse on AOP and OP levels. Our approach included the use of UV-detectable amounts of selected synthetic peptide standards to establish the HPLC methodology, and to establish procedures to minimize peptide loss during sample preparation. We used HPLC mobile phases

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which were totally volatile so that HPLC-fractionated samples would be compatible with quantitation by radioimmunoassay. We extended the approaches of Bhathena et al. [3], who combined the use of protease inhibitors, Sep-Pak extraction, and various solvent conditions to isolate β-endorphin, leu-enkephalin, and met-enkephalin from plasma. Although others [4–6] showed that HPLC can be used to separate several neuropeptides, we intended to resolve an extended series of enkephalins, dynorphins, and β-endorphins using conditions which would maximize their recovery from biological fluids and allow for quantitation by radioimmunoassay.

## 2. Experimental

### 2.1. Chemicals and supplies

($Tyr^0$)-melanocyte-stimulating hormone-release inhibiting factor [($Tyr^0$)-MIF-I] was obtained from Bachem (Philadelphia, PA, USA). Dynorphin A (porcine), dynorphin A 1–8 (porcine), dynorphin A 1–9 (porcine), dynorphin A 1–12 (porcine), dynorphin A 1–13 (porcine), dynorphin B (porcine), big dynorphin (porcine), big dynorphin 1–24 (porcine), β-endorphin 1–27 (human), cholecystokinin octapeptide, substance P, α-melanocyte stimulating hormone, and morphine modulating neuropeptide were obtained from Peninsula Laboratories (Belmont, CA, USA). [$Met^5$]-Enkephalin, [$Leu^5$]-enkephalin, β-endorphin (β-lipotropin 61–91; human) were obtained from Cambridge Research Biochemicals (Wilmington, DE, USA). [$Met^5$]-Enkephalin was stored at −80°C as a 10 mg/ml solution in water, and the others were stored as 1 mg/ml solutions in water.

Peptidase inhibitor cocktail was prepared by mixing 1 ml of 3 mg/ml EDTA-disodium salt, 1 ml of 46 m*M* citric acid, 10 μl of 23 trypsin inhibitor units (TIU)/ml aprotinin (Sigma, St. Louis, MO, USA) and 2 μl of 25 mg/ml of Ubenimex (bestatin; Peptides International, Louisville, KY, USA) in dimethyl sulfoxide. Phosphate buffered saline (PBS, pH 7.4) contained 0.1 *M* phosphate, 0.05 *M* NaCl, 0.1% Triton X-100, and 0.01% $NaN_3$. Water was provided from a MilliQ UV Plus system (Millipore, Bedford, MA, USA). Other reagents and solvents were analytical or HPLC grade, as appropriate.

Frozen, outdated human plasma was kindly provided by the Francis Scott Key Medical Center Blood Bank (Baltimore, MD, USA). The plasma was thawed, transferred to a polypropylene beaker, and with stirring, 1/10th volume of 1 *M* HCl was added. One milliliter portions were placed into CryoTubes (Nunc, Naperville, IL, USA) and stored frozen at −80°C.

### 2.2. Reversed-phase HPLC resolution of synthetic AOPs and OPs

Synthetic peptide standards were diluted in water to final concentrations of 2.5–5 μg/500 μl, and 420 μl were injected onto a Waters Model 510 HPLC (Milford, MA, USA) for retention time determination. The mobile phase was a 70-min linear gradient of 22.5% to 56.2% aqueous acetonitrile, in the presence of 0.1% aqueous trifluoroacetic acid (TFA). Separations were achieved on a Phase II (ODS, Bioanalytical Systems, West Lafayette, IN, USA) column (250 × 4.6 mm I.D.) and was packed with 5-μm particles. The flow-rate was 1 ml/min. UV absorbance at 215 nm was monitored by a Waters 490E detector. Peptides in mixtures were identified by comparing their individual retention times. In some experiments, fractions were collected every thirty seconds for 1 h in polypropylene tubes (Sarstedt, Newton, NC, USA). At the end of the run, tubes were removed, capped (Sarstedt), and stored at −80°C.

In some cases, pooled fractions termed peaks I and II from the first HPLC run were further resolved by a second HPLC run. Fractions containing the desired peptide material were pooled, vacuum centrifuged to dryness, resuspended in 20 μl of ethanol–acetic acid–water (90:4:6, v/v), and then diluted to 300 μl with water. Aliquots of 280 μl were injected onto the HPLC system. Chromatography was carried out on a WP MOS $C_8$ column (150 × 4.6 mm I.D., 5

μm particle size) obtained from Alltech (Deerfield, IL, USA) at a flow-rate of 1 ml/min. A linear gradient of 19.5% to 27.2% aqueous acetonitrile containing 0.1% TFA was applied in 40 min.

### 2.3. Sample preparation for Sep-Pak extraction

Sep-Pak $C_{18}$ cartridges (Waters) were prewetted with 5 ml of acetonitrile, followed by 5 ml of 0.1% aqueous TFA. In order to compare the results with those obtained after plasma extraction 100 μl of 1 *M* HCl and 145 μl of a peptidase inhibitor cocktail were added first, followed by 1 ml of 0.1% aqueous TFA containing 1 μg each of various synthetic peptides and another 10 ml of 0.1% aqueous TFA. The sample was taken into a plastic syringe and loaded onto a prewetted cartridge equipped with a 26 gauge, 1.58 cm needle attached to the other end. The cartridge was washed twice with 10 ml of 0.1% TFA, and then eluted with 6 ml of solvent as specified in the results section. The eluate was collected in a 50-ml polypropylene tube and stored at −80°C until further analysis.

In a later experiment, 1-ml samples of frozen acidified plasma were thawed in the presence of 145 μl of peptidase inhibitor cocktail (see above) at room temperature and placed on ice. The sample was added to 1 ml of 0.1% TFA containing 1 μg each of various synthetic peptides, and then 10 ml of 0.1% TFA was added. The mixtures were loaded onto and eluted from Sep-Pak cartridges as described above, and the eluates stored at −80°C.

### 2.4. HPLC resolution of Sep-Pak eluates

Frozen Sep-Pak eluates were evaporated to dryness in a Savant Speed-Vac (Farmingdale, NY, USA) vacuum centrifuge. The dried sample was resuspended in 100 μl of ethanol–acetic acid–water (90:4:6, v/v). After five minutes 400 μl of water was added and the entire sample was passed through a 0.2-μm syringe filter (Supelco, Bellefonte, PA, USA). The filtrate was loaded into a glass insert for HPLC injection by a WISP autoinjector (Waters).

## 3. Results

As Fig. 1A shows, a single HPLC run is sufficient to resolve most of the 17 test peptides. However, some of the peptides co-eluted. Therefore, a second HPLC run was developed to resolve these co-eluting peptides. Peak groups were pooled and subjected to an additional HPLC run, as shown in Fig. 1B, which resolved these peptides. Retention times are shown in Table 1.

Table 2 compares the results of using two different solvents to elute Sep-Pak cartridges that had been loaded with mixtures of four synthetic peptides. The use of aqueous acetonitrile–0.1% aqueous TFA as a solvent for Sep-Pak elution enabled higher than 90% recovery for each of the four test peptides, as analyzed by HPLC with UV detection. Aqueous acetonitrile–0.1% TFA was clearly superior to ethanol–acetic acid–water for recovering peptides from Sep-Pak cartridges.

After the aqueous acetonitrile–0.1% TFA solution was used to elute peptide mixtures from Sep-Pak cartridges, the eluates were dried in a vacuum centrifuge. Then, various solvents were compared for their ability to redissolve the four test peptides from dryness, as analyzed by HPLC with UV detection. Table 3 shows that ethanol–acetic acid–water (90:4:6, v/v) was the best of nine solvent mixtures tested, enabling higher than 75% recovery. In contrast, phosphate-buffered saline, which is often used for resuspension of samples for RIA, was unsatisfactory as a solvent for redissolving dynorphin and β-endorphin peptides.

Next, the effect of plasma on the recovery of 15 synthetic peptides was investigated. Peptide mixtures were added to 1 ml of 0.1% TFA or to 1 ml of plasma, 10 ml of 0.1% TFA was added, and the mixtures loaded onto Sep-Pak cartridges. In the same way as for the experiment described in Table 3, the Sep-Pak cartridges were eluted with aqueous acetonitrile–0.1%

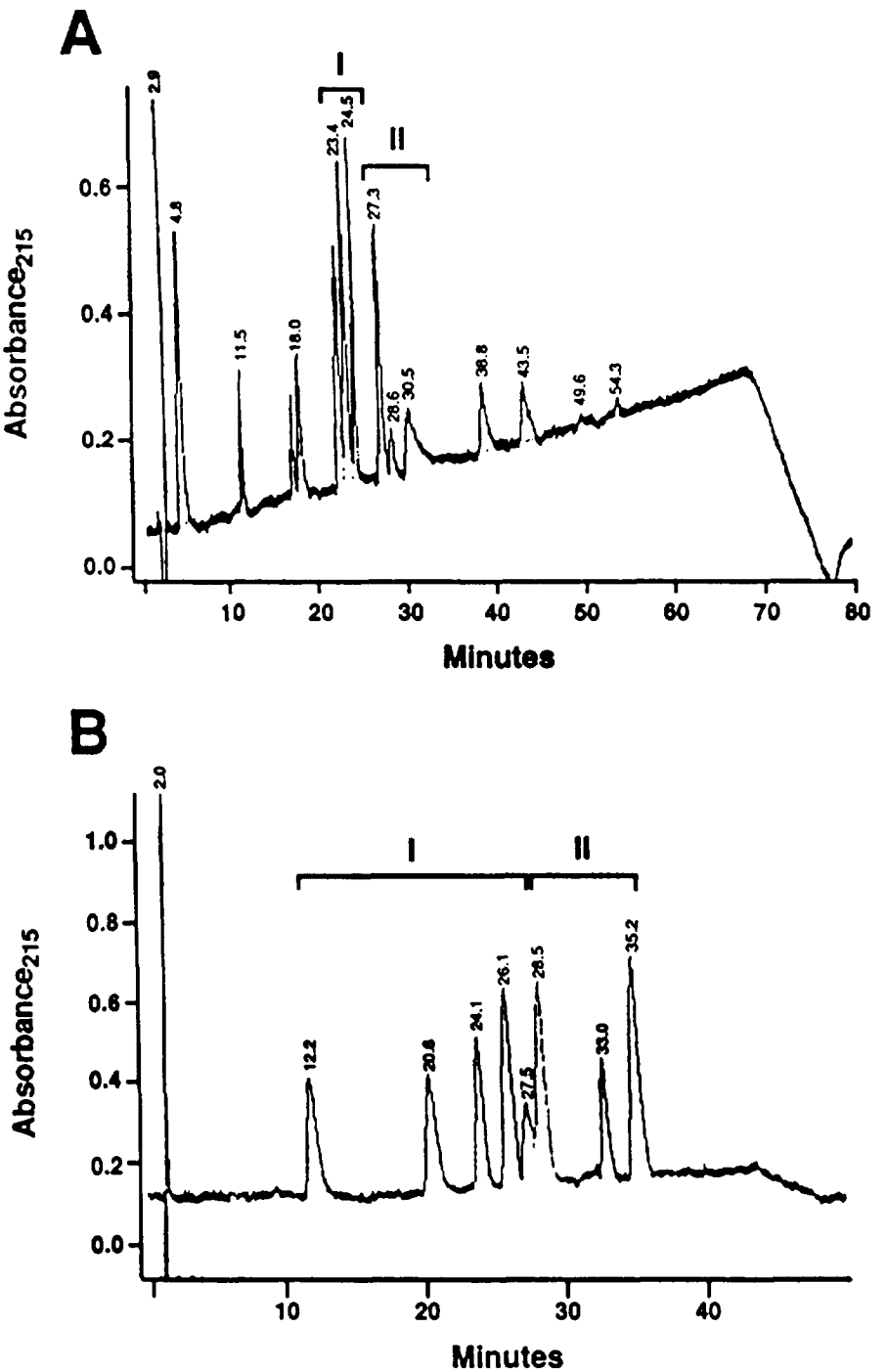


Fig. 1. HPLC resolution of synthetic AOPs and OPs. As described in the text, solutions of synthetic peptides were injected onto the HPLC column and monitored by UV for retention time determination. Fractions containing peaks I and II (Fig. 1A) were pooled and subjected to an additional HPLC run (Fig. 1B), which resolved these peptides. Peaks are identified according to retention time in Table 1.

Table 1
HPLC resolution of opioid and anti-opioid peptides

| Peptide | Retention time (min) |
|---|---|
| *First run* | |
| Tyrosine-MIF | 4.8 |
| Substance P | 4.8 |
| [Met$^5$]-Enkephalin | 11.5 |
| [Leu$^5$]-Enkephalin | 17.2 |
| Dynorphin A 1–9 | 17.2 |
| REGION I | |
| Dynorphin A 1–13 | 22.0 |
| α-Melanocyte stimulating hormone | 22.0 |
| Dynorphin A 1–8 | 23.4 |
| Dynorphin B | 23.4 |
| Cholecystokinin octapeptide | 24.5 |
| REGION II | |
| Dynorphin A | 27.3 |
| Dynorphin A 1–12 | 28.6 |
| Morphine modulating neuropeptide | 30.5 |
| Big dynorphin | 38.8 |
| Big dynorphin 1–24 | 43.5 |
| β-Endorphin | 49.6 |
| β-Endorphin 1–27 | 54.3 |
| *Second run* | |
| Dynorphin A 1–9 | 12.2 |
| Dynorphin A 1–8 | 20.6 |
| α-Melanocyte stimulating hormone | 24.1 |
| Dynorphin B | 26.1 |
| Dynorphin A 1–13 | 27.5 |
| Morphine modulating neuropeptide | 28.5 |
| Dynorphin A 1–12 | 33.0 |
| Dynorphin A | 35.2 |

TFA. The eluates were dried, resuspended in ethanol–acetic acid–water, and analyzed by HPLC with UV detection. Chromatograms describing this separation in plasma extracts are shown in Fig. 2. Table 4 shows that, in the absence of plasma, over 70% of each of the 15 peptides was recovered. In the presence of plasma, the recovery of one of the peptides, big dynorphin, was reduced to 44%, and the recoveries of five others were not determined because of interference from unknown peaks in plasma samples that co-eluted with the synthetic peptides. Nevertheless, recoveries of the remaining nine peptides were over 70%.

## 4. Discussion

We developed conditions to enable the use of HPLC for the simultaneous resolution of microgram amounts of multiple neuropeptides, and their recovery, pooling, and storage for subsequent analysis. Initially, we encountered difficulties because the "stickiness" of these peptides onto surfaces of tubes led to substantial losses. Therefore, we determined the experimental conditions to limit losses of these peptides in preparation for HPLC. The HPLC method effected the separation of 17 synthetic neuropeptides with good recovery. We found that the resolution and recovery of most of the synthetic peptides was not affected when added to plasma. However, recoveries of tyrosine-MIF, substance P, [Leu$^5$]-enkephalin, dynorphin A (1–9), and big dynorphin (1–24) in plasma extracts could not be calculated due to matrix interferences, whereas the recovery of big dynorphin was substantially decreased (44 versus 80% in 0.1% aqueous TFA alone). The use of volatile solvents in this procedure makes HPLC-fractionated samples amenable to quantitation by radioimmunoassay, thus allowing the simultaneous quantitation of multiple AOPs and OPs. By combining the resolving power of HPLC with the sensitivity of RIA, the work in this study may be extended to investigate concentrations of AOPs and OPs in body fluids such as plasma and cerebrospinal fluid.

Although others have studied neuropeptide concentrations in various tissues and body fluids, as far as we know we are the first to have effected, in the same HPLC run, separation of enkephalins, dynorphins, and β-endorphins. Whereas a second HPLC run was required to distinguish among the dynorphins, α-MSH, and morphine-modulating neuropeptide, we are not aware of any other studies that have effected HPLC separation of such an extensive an assortment of peptides as in our study. For example, Venn [5] used HPLC to effect good separation

Table 2
Comparison of solvents for elution of selected opioid and anti-opioid peptides from Sep-Pak cartridges

| Solvent | Recovery (%) | | | |
|---|---|---|---|---|
| | [Met$^5$]-Enkephalin | Dynorphin A | Big dynorphin 1–24 | β-Endorphin |
| Acetonitrile–TFA–water (60:0.1:39.9) | 99 | 96 | 91 | 96 |
| Ethanol–acetic acid–water (90:4:6) | 97 | 58 | 36 | 70 |

Mixtures containing 1 μg of each synthetic peptide were loaded onto Sep-Pak cartridges and eluted with 6 ml of solvent. The eluates were vacuum centrifuged to dryness, resuspended in 20 μl of ethanol–acetic acid–water (90:4:6), diluted to 400 μl with water, and injected onto the HPLC column. Values are percentage recovery of each peptide relative to a portion of the peptide mixture that had been injected directly, and are the means of duplicate determinations, which did not vary by more than 10%. The experiment was repeated once with similar results.

between β-endorphin and β-lipotropin, but many of the neuropeptides used in this investigation were not considered.

Various researchers have combined HPLC separation with immunological assays to study the effect of various conditions on neuropeptide concentrations, e.g. their dependence on aging, drug treatment, and surgery (see below). For

Table 3
Comparison of solvents for redissolving selected opioids and anti-opioids from dried Sep-Pak eluates

| Solvent | Recovery (%) | | | |
|---|---|---|---|---|
| | [Met$^5$]-Enkephalin | Dynorphin A | Big dynorphin 1–24 | β-Endorphin |
| Ethanol–acetic acid–water | | | | |
| 90:4:6 | 87 | 98 | 96 | 77 |
| 20:4:76 | 106 | 96 | 10 | 107 |
| Water | 78 | 83 | 46 | 13 |
| 0.1 *M* PBS pH 7.4 | 84 | 0 | 0 | 0 |
| 0.1 M PBS pH 7.4 + 0.1% Triton X-100 | 84 | 0 | 0 | 0 |
| 0.1% TFA | 54 | 0 | 0 | 0 |
| Methanol–TFA–water | | | | |
| 20:0.1:79.9 | 78 | 58 | 0 | 0 |
| 99.9:0.1:1.0 | 72 | 53 | 0 | 0 |
| 0.1 *M* Acetic acid | 98 | 92 | 6 | 93 |

Mixtures containing 1 μg of each synthetic peptide were loaded onto Sep-Pak cartridges, and eluted with 6 ml of acetonitrile–TFA–water (60.0:0.1:39.9). The eluates were vacuum centrifuged to dryness, resuspended in 20 μl of the solvents listed above, diluted to 400 μl with water, and injected onto the HPLC column. Values are percentage recovery of each peptide relative to a portion of the peptide mixture that had been directly injected, and are the means of duplicate determinations, which did not vary by more than 10%. The experiment was repeated once with similar results.

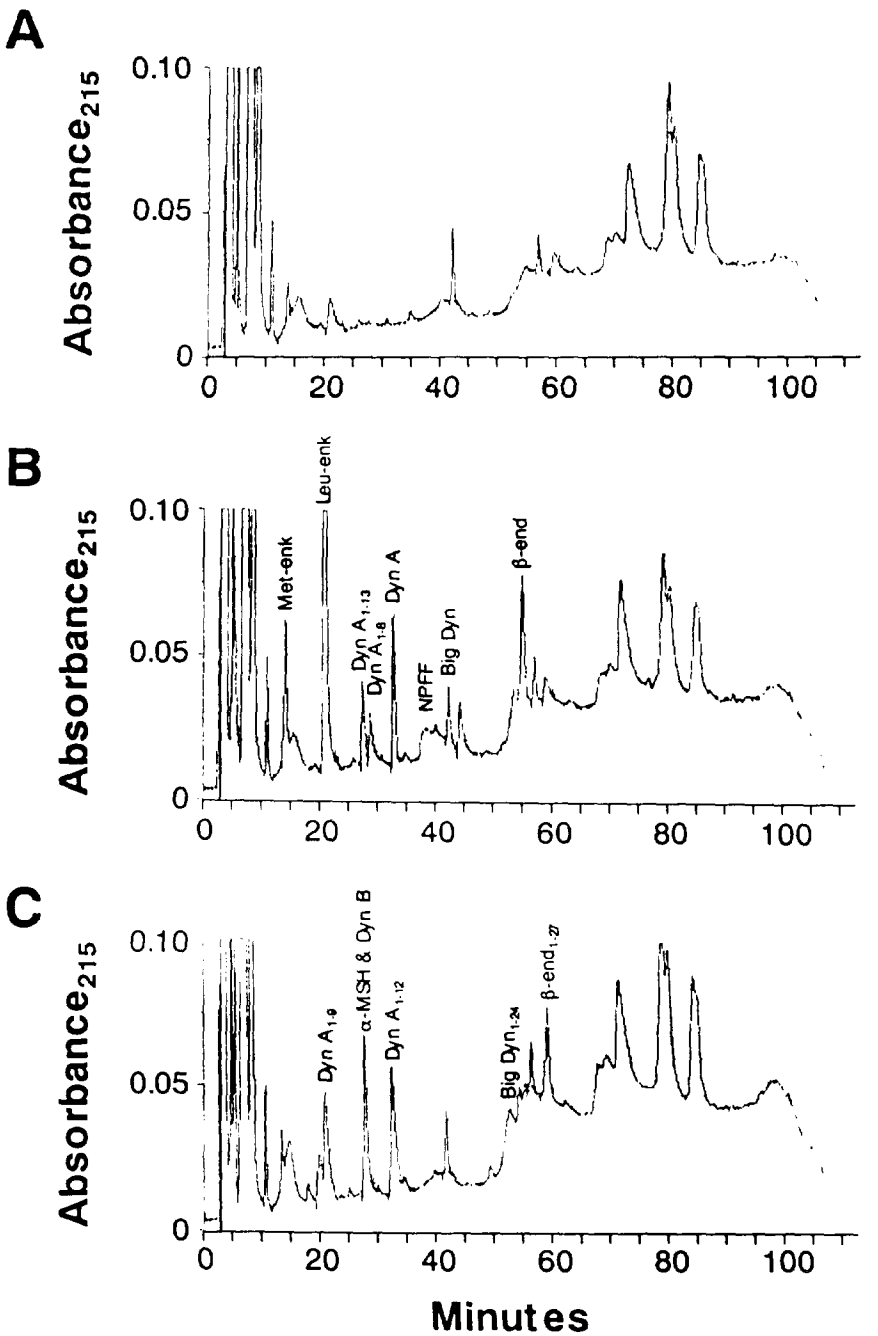


Fig. 2. HPLC resolution of synthetic AOPs and OPs from human plasma. Aliquots of human plasma without and with added synthetic peptides were extracted as described in the Experimental section using acetonitrile–TFA–water (60:0.1:39.9, v/v) for Sep-Pak elution and ethanol–acetic acid–water (90:4:6, v/v) for resolvation prior to HPLC analysis. (A) One milliliter of plasma was processed as described above. (B) A 2-μg amount of tyrosine-MIF, 2.5 μg each of dynorphin A 1–13, dynorphin A 1–8, dynorphin A, morphine modulating neuropeptide, and big dynorphin, and 5 μg each of Leu$^5$-enkephalin, Met$^5$-enkephalin, and β-endorphin were added to 1 ml of human plasma and processed as described above. (C) A 2.5-μg amount of dynorphin A 1–9, α-MSH, and β-endorphin 1–27, and 5 μg each of substance P, dynorphin A 1–12, and dynorphin B were added to 1 ml of human plasma and processed as described above.

Table 4
Effect of plasma on recovery of various opioid and anti-opioid peptides

| Peptide | Without plasma | With plasma |
|---|---|---|
| Tyrosine-MIF | 71 | ND |
| Substance P | 99 | ND |
| [Met$^5$]-Enkephalin | 92 | 92 |
| [Leu$^5$]-Enkephalin | 98 | ND |
| Dynorphin A 1–9 | 100 | ND |
| Dynorphin A 1–13 | 102 | 84 |
| $\alpha$-Melanocyte stimulating hormone | 85 | 72 |
| Dynorphin A 1–8 | 108 | 82 |
| Dynorphin B | 103 | 111 |
| Dynorphin A | 96 | 86 |
| Dynorphin A 1–12 | 99 | 82 |
| Big dynorphin | 80 | 44 |
| Big dynorphin 1–24 | 95 | ND |
| $\beta$-Endorphin | 99 | 86 |
| $\beta$-Endorphin 1–27 | 80 | 94 |

Synthetic peptide mixtures were added to aqueous 0.1% TFA or to plasma, and processed as described in Table 3. Dried Sep-Pak eluates were resuspended in ethanol–acetic acid–water (90:4:6) for HPLC analysis. Values are percentage recoveries relative to the peptides in the mixture that have been directly injected and are the means of duplicate determinations, which did not vary by more than 10%. The experiment was repeated once with similar results. ND means not determined because the synthetic peptide co-eluted with an unknown peak from the plasma sample.

example, Dax et al. [7] concluded that endorphin and dynorphin concentrations do not change progressively with aging in rat pituitary and hypothalamus. Fullerton et al. [8] found that the dynorphin content of the male rat pituitary anterior lobe decreases on castration, and this change can be reversed by dihydrotestosterone administration. Ho and Wen [9] combined Sep-Pak extraction, HPLC, and a receptor-binding assay to provide evidence for an hitherto structurally unidentified, opioid-like component in human cerebrospinal fluid that is released during treatment of pain by electroacupuncture.

Further experiments on the development of an appropriate HPLC–RIA coupling technique are planned in order to extend our work to the study of the effects of drug use on AOPs and OPs in plasma and cerebrospinal fluid.

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