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Note

Analysis of peptides in tissue and plasma

Use of silicic acid extraction and reversed-phase columns for rapid purification prior to radioimmunoassay

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Since the discovery of the endogenous opiate peptides, met- and leu-enkephalin [1] and the endorphins [2, 3], many workers have developed assay systems for the measurement of these compounds in tissue and in plasma. Both radioimmunoassay (RIA) [4—7] and high-performance liquid chromatography (HPLC) [8] or HPLC with bioassay or receptor assay [9, 10] have been used effectively to quantitate these peptides. When large scale studies are undertaken which generate many samples, RIA is the method of choice as hundreds of samples can be processed in a working day. Because antigen—antibody binding is affected by the presence of impurities in the incubation mixture, adequate sample purification prior to RIA is necessary in order to obtain accurate data. Gay and Lahti [11] have recently reported the use of Sep-Pak reversed-phase cartridges for the separation of radioactive and synthetic enkephalins and endorphins. We have developed two simple rapid procedures for sample preparation, one which uses the reversed-phase Sep-Pak cartridges to coelute endogenous met-enkephalin, leu-enkephalin, and β-endorphin from rat brain tissue extracts and the other for plasma which uses silicic acid extraction. The peptides are co-eluted in a single sample for convenience and the procedure is suitable in situations in which the antibodies employed in the RIA are specific for each peptide.

MATERIALS AND METHODS

Brain parts were sonicated in 1 ml 0.2 *N* hydrochloric acid—acetone (25:75)

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containing phenylmethylsulfonyl fluoride, an enzyme inactivator (0.2 mg/ml) and pentachlorophenol (0.02 mg/ml) in Eppendorf plastic tubes. After centrifuging at 12,800 *g* in a Brinkman Eppendorf centrifuge, the supernatant was decanted into a silanized glass 12 × 75 mm test tube, the pellet resuspended in 0.75 ml hydrochloric-acid—acetone, and centrifuged. The supernatants were combined and dried under nitrogen until almost dry. The drying was completed in a vacuum oven. The residue was taken up in 2.0 ml quartz-distilled water and allowed to incubate for 1 h at 4°C.

Sep-Pak reversed-phase C_{18} cartridges (Waters Assoc., Milford, MA, U.S.A.) were used for adsorption of the peptides. These cartridges were used with a Vac-Elut (Analytichem International, Harbor City, CA, U.S.A.) — an evacuated metal box with 10 ports on the top lid. One end of the C_{18} cartridge was inserted into a plastic pipette tip (1—200 µl size, Robbins Scientific Corp., Mountain View, CA, U.S.A.), which in turn went into a port. The other end of the cartridge was attached to a 3-ml plastic disposable syringe. If a Vac-Elut is unavailable, the sample and reagents may be pushed through by means of the syringe plunger. The C_{18} reversed phase was activated before sample application by passing through it 2 ml acetone followed by 5 ml quartz-distilled water. Glass wool was placed in the bottom of the syringe to filter the sample before it reached the C_{18} cartridge. Following sample application, the cartridges were washed twice with 2 ml 4% acetic acid, and met- and leu-enkephalin and β-endorphin were eluted in 1.5 ml 0.2 *N* hydrochloric acid—actone (25:75). The eluate was dried under nitrogen in a vacuum oven and the residue taken up in an appropriate volume of RIA buffer.

The cartridges may be regenerated and used again. After sample elution an additional 3 ml of the hydrochloric acid—acetone was passed through and the cartridges were stored in methanol. Before use, acetone and water were passed through as described above.

Plasma may be applied directly onto the cartridge, but when only β-endorphin-like immunoreactivity was to be measured by RIA, another method that is both rapid and economical was employed. A round disk of filter paper was placed in the bottom of a 3-ml plastic disposable syringe, wetting the paper slightly to keep it in place. Dry silicic acid (Bio-Sil A, 200—400 mesh, Bio-Rad Labs., Richmond, CA, U.S.A.) was added to the 1.5-ml mark using a small plastic dropper bottle and the syringes put directly into the ports of the Vac-Elut. The silicic acid was well wetted with quartz-distilled water. Plasma (1.5 ml) was adjusted to pH 4.0 with 100 µl 1 *N* hydrochloric acid and poured onto the silicic acid. The silicic acid was washed twice with 1.5 ml quartz-distilled water followed by 1.5 ml 1 *N* hydrochloric acid using wash bottles. β-Endorphin was eluted with 2.0 ml 50% acetone. The eluate was taken to dryness under nitrogen and vacuum and the residue reconstituted in RIA buffer. The syringes were discarded after use.

RESULTS AND DISCUSSION

Recovery was monitored by adding iodinated peptide to either brain extracts or plasma and was found to be quite high, 96—98% for all peptides with the exception of iodinated β-endorphin where the recovery was 88%. The S.E.M.

of recoveries for leu- and met-enkephalin and β-endorphin were under 1%. In order to determine whether brain tissue concentration affected recovery, various amounts up to 1 g were extracted with hydrochloric acid—acetone, dried, and passed through the cartridges. The recoveries of the three peptides remained unchanged with the S.E.M. still under 1%. Similarly, cold peptide in concentrations to 1 mg was added to tissue extracts and adsorbed onto the reversed phase with no alteration in recovery. Iodinated β-lipotropin (β-LPH) and dynorphin(1—13) were also adsorbed onto and eluted from these columns with similar results. Approximately 10% of the β-endorphin adsorbed to the silanized glass test tube when the sample was applied to the silicic acid or C_{18}. The β-endorphin was recovered by putting the hydrochloric acid—acetone or 50% acetone into the tube and vortexing well before eluting.

Silicic acid has been used for the extraction of β-endorphin [7] and adrenocorticotrophin [12] from plasma. These procedures involve time consuming end-to-end shaking of the sample with silicic acid, followed by centrifugation and more shaking to elute. Using the column method described above, ten samples can be processed through either column in approximately 5 min with an improved recovery. Table I shows the levels of met- and leu-enkephalin and

TABLE I

PEPTIDE CONCENTRATIONS IN VARIOUS REGIONS OF RAT BRAIN

Five male adult rats were decapitated, their brains removed, and dissected into the areas shown. Protein was determined by the method of Lowry et al. [13]. Data are given in pmol/mg protein ± S.E.M.

Brain area	Met-Enkephalin	Leu-Enkephalin	β-Endorphin
Caudate	12.6 ± 0.95	1.85 ± 0.22	
Hypothalamus	8.21 ± 0.39	2.41 ± 0.56	0.61 ± 0.04
Septum	3.66 ± 0.44	0.62 ± 0.11	0.06 ± 0.01
Brain Stem	2.49 ± 0.08	0.61 ± 0.05	
Mid Brain	2.03 ± 0.09	0.53 ± 0.04	0.03 ± 0.005
Cortex	1.76 ± 0.16	0.25 ± 0.03	
Hippocampus	1.10 ± 0.08	0.20 ± 0.01	
Cerebellum	0.96 ± 0.04	0.12 ± 0.04	

β-endorphin-like immunoreactivity measured by RIA in various regions of rat brain using the reversed-phase C_{18} separation procedure. Using silicic acid extraction, human β-endorphin-like immunoreactivity was equivalent to approximately 6—31 fmol human β-endorphin per ml of plasma. Because the antibody used in the RIA is directed against the C-terminal portion of β-endorphin and crossreacts 100% on a molar basis with β-LPH, these concentrations represent β-LPH as well as β-endorphin-like immunoreactivity.

Serial dilutions of tissue extracts were superimposable on the standard RIA curves of each peptide. A typical met-enkephalin RIA standard curve is shown in Fig. 1 with several dilutions of rat brain caudate, cortex, and midbrain samples.

The silicic acid and reversed-phase column procedures as described are useful for rapid purification of peptides in which the assay will be performed

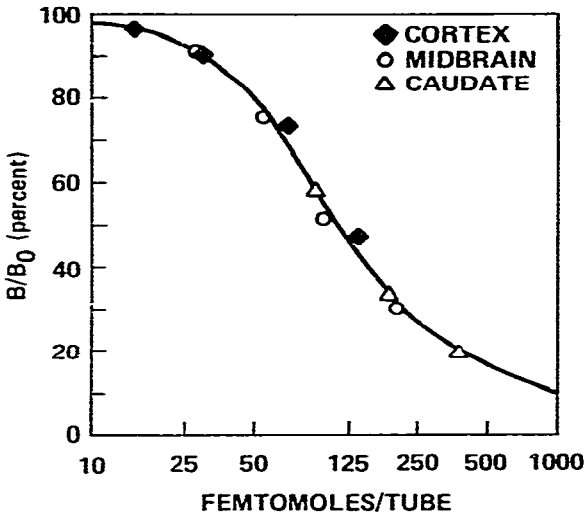

Fig. 1. Comparison of RIA standard curve for met-enkephalin and serial dilutions of various brain region extracts. Procedure as described in text. B/B_0 is binding of ^{125}I-labelled met-enkephalin in the presence of competing peptide compared to binding of ^{125}I-labelled met-enkephalin alone, expressed as percent.

with characterized and specific antibodies. We have demonstrated that the method allows collection from biological samples of various opioid peptides and it can be expected that other opioid and non-opioid peptides are obtained in the collected fraction. Thus, the usefulness of these coelution purification procedures will depend upon the availability of highly specific antibodies. In situations in which such antibodies are not available, or in other situations in which there is reason to believe there may be known or unknown substances which would significantly crossreact with the available antibodies, other more specific isolation procedures will be necessary. Gradient elution using these or related reversed-phase columns may be helpful in such situations. Within the confines of a situation for which it is suited, the described methods are rapid, reproducible, facilitate processing of large numbers of samples, and are easily used for purification of peptides from tissue samples preparatory to specific assay.

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