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Research Article

Determination of β-Endorphin and Fragments Thereof in Human Plasma Using High-Performance Liquid Chromatography and a Multiple Radioimmunoassay System

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A method for the determination of β-endorphin and β-endorphin fragments in human plasma was developed. β-Endorphin-related peptides were extracted from plasma using octadecasilyl-silica cartridges. Extracts were subjected to reversed-phase high-performance liquid chromatography (HPLC). Extracts as well as HPLC column eluates were assayed using a multiple radioimmunoassay system; several antibodies directed against various distinct regions of the β-endorphin molecule were employed. Using this method, evidence for the presence of multiple β-endorphin fragments in the plasma of healthy young volunteers (under normal conditions) was obtained.

KEY WORDS: β-endorphin in human plasma; multiple β-endorphin immunoreactive materials; HPLC of peptides; multiple radioimmunoassay system; peptide region specific radioimmunoassays.

INTRODUCTION

β-Endorphin immunoreactivity levels in human plasma under "normal" or "stress" conditions or in certain disease states have been described by many investigators (1–4); however, the data obtained are extremely controversial, e.g., normal plasma levels of β-endorphin immunoreactive materials have been reported to vary from 0 to about 600 fmol/ml plasma (5). Individual variability of the plasma donors (6,7) as well as different methods employed might be considered to be responsible for this discrepancy of data.

Moreover, besides β_h-endorphin[2] and β_h-LPH, further β_h-endorphin-related peptides seem to be present in the plasma (1,2,8). However, by conventional methods for separation and demonstration of the immunoreactive materials, e.g., gel filtration or just one antibody recognizing a certain segment of the β_h-endorphin or β_h-LPH molecule, a detailed characterization of the immunoreactive materials was not possible.

Thus, we developed a novel method for determination of β_h-endorphin as well as fragments thereof, replacing conventional extraction methods by octadecasilyl-silica cartidge extraction, gel filtration by reversed-phase HPLC and the single-antibody RIA by a multiple RIA system including a region-specific RIA for β_h-endorphin.

MATERIALS AND METHODS

Reagents

β_h-LPH was a generous gift of Dr. C. H. Li, San Francisco. β_h-Endorphin (1–9) was obtained from Peninsula, Belmont; β_h-endorphin (1–16), β_h-endorphin (1–31), β_h-endorphin (27–31), and β_c-endorphin (1–31) were from Bachem, Bubendorf, Switzerland. [Leu]Enkephalin and [Met]enkephalin and all di- and tripeptides used were purchased from Serva, Heidelberg, FRG. Degradation during storage was monitored in our laboratory using reversed-phase HPLC.

Amino acids, thyroglobulin, chloramine T, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, Triton X-100, and thimerosal were obtained from Serva, Heidelberg, FRG; bovine serum albumin and sodium metabisulfite were from Sigma, Taufkirchen, FRG; Freund's adjuvant was from Behringwerke, Marburg, FRG; and acetonitrile (HPLC grade) was from Roth, Karlsruhe, FRG. All other reagents were analytical-reagent grade from Merck, Darmstadt, FRG.

Sample Collection

All syringes and tubes used for sample collection and subsequent extraction were made out of polypropylene (Sarstedt, Nürmbrecht, FRG); all procedures were carried out at 0 to 4°C.

Blood samples were drawn into disposable syringes and immediately transferred to chilled tubes containing sodium EDTA (1 mg/ml blood). Blood was centrifuged (1000*g*, 15 min), and plasma was carefully separated, subsequently acidified with 1 *N* HCl (100 μl/ml plasma), and centrifuged

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[2] Abbreviations used: RIA, radioimmunoassay; HPLC, high-performance liquid chromatography; β_h-endorphin/β_h-E, human β-endorphin; β_h-LPH, human β-lipotropin; β_c-endorphin/β_c-E, camel β-endorphin; [Leu]enk, [Leu]enkephalin; [Met]enk, [Met]enkephalin.

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(20,000*g*, 10 min) to remove precipitates; the supernatant was extracted immediately.

Extraction

Up to 20 octadecasilyl-silica cartridges (SEP PAK C18 cartridges, Waters Associates, Milford, Conn.) mounted on a rack were used for the extraction procedure. Samples and solvents were brought through the cartridge using negative pressure. The flow rate was approximately 1 ml/min.

Cartridges were prepared for extraction by the application of, in succession, 5 ml methanol, 5 ml 6 *M* urea, and 10 ml distilled water; then 5-ml aliquots of the acidified plasma supernatant were applied to each cartridge, followed by 10 ml distilled water and 10 ml 4% acetic acid (9). Retained peptides were eluted with 5 ml of a mixture of 1-propanol and acetic acid (96/4; v/v).

The organic solvent of the eluate was evaporated using a speed vac concentrator (Savant, New York); the remaining aqueous phase was lyophilized and the dried residue, henceforth referred to as "extract," was stored at −35°C.

For RIA, the extracts were reconstituted in buffer 1 (see below); for HPLC, the extracts were reconstituted in solvent B, filtered, adjusted to initial conditions of the HPLC gradient, filtered again, and applied to the column (for HPLC solvents and equipment, see below).

To examine the recoveries of β_h-LPH, β_h-endorphin, and fragments thereof, labeled or unlabeled peptides were added to acidified plasma supernatant and extracted. Extracts were either directly reconstituted in buffer 1 for RIA or reconstituted in HPLC solvent B, filtered, adjusted to initial conditions (HPLC gradient), filtered again, dried, and reconstituted in buffer 1 for RIA.

To ensure linear recovery, correlation coefficients were calculated for a linear relationship between the amounts of peptides actually extracted and the corresponding amounts to be expected for 100% recovery. The linearity was tested for peptide concentrations in the plasma supernatant ranging from 2 up to 500 fmol/ml.

The intrassay coefficient of variation for the extraction method was calculated from the recovery values of 10 simultaneously extracted samples from a plasma supernatant pool.

The interassay coefficient of variation was calculated from the recovery values of 10 samples of a plasma supernatant pool extracted at different times.

HPLC

The HPLC system used consisted of Waters Associates equipment (Milford, Conn.) including two solvent delivery systems (Model 6000 A), a solvent programmer for gradient elution (Model 660), and a universal liquid chromatography injector (Model U6K); a variable-wavelength monitor (Pye Unicam, Cambridge, UK) and a three-channel chart recorder (BBC-Goerz, Nürnberg, FRG) were attached. For reversed-phase separation a μBondapak C18 column (3.9 × 300 mm, Waters Associates, Milford, Conn.) without a guard column was employed. The mean theoretical plate number for β_h-endorphin (1–31) calculated in our laboratory was about 7500 plates/column at ambient temperature. The following solvents were used: the aqueous solvent A was 0.01 *N* HCl (pH 2.00) (10); the organic modifier, solvent B, was 60% acetonitrile in water (v/v) containing L-arginine, L-histidine, and L-tryptophan (50 μ*M* each). Acetonitrile was HPLC grade; water was double-distilled. All solvents were filtered through 0.5-μm Fluoropore filters (Millipore, Bedford, Mass.) prior to use and degassed by heat, vacuum, and ultrasonication.

HPLC was carried out at an ambient temperature of 20–22°C. Equilibration of the column with the initial-conditions solvent mixture (see below) before starting gradient elution was monitored using the variable-wavelength device at 215 nm.

For chromatography, up to 20 extracts were dissolved in solvent B, pooled (maximum volume, 300 μl), and filtered through a 0.5-μm Fluoropore filter (sample clarification kit, Millipore, Bedford, Mass.). The filtrate was adjusted to initial conditions of gradient elution (see below) with solvent A (maximum volume, 1.7 ml) and filtered again through a 0.5-μm Fluoropore filter; this procedure was employed to prevent possible blockade of the column inlet by particles as well as precipitation of extract material under gradient elution conditions. The maximum sample volume injected was 1800 μl; the rest, usually of 200 μl, were dried using the Speed Vac concentrator and reconstituted in buffer 1 for RIA.

A linear continuous gradient was employed, varying within 50 min from solvents A/B (85/15%) (initial conditions) to solvents A/B (10/90%) (final conditions) at a flow rate of 1 ml/min. One-milliliter fractions of the eluate were collected in polyethylene tubes (Sarstedt, Nürmbrecht, FRG), dried using a Speed Vac concentrator, and reconstituted in buffer 1 for RIA.

To ensure that the column did not contribute peptides from a preceding chromatography, prior to each sample run gradient elution without sample injection was performed and the eluate was assayed by RIA. The loading manifold was routinely washed before sample injection.

For determination of retention times synthetic peptides were chromatographed either directly or after extraction from plasma. For determination of recovery rate and resolution, synthetic peptides extracted from plasma supernatant were injected, each in an amount of 5000 fmol. The resolution of two peptides separated by HPLC was calculated from $R_s = 2 \cdot [(t_2 - t_1)/(w_1 + w_2)]$, where t_1 and t_2 are the retention distances and w_1 and w_2 are the peak base widths of peak 1 and peak 2, respectively. For calculation of R_s mean values of t_1, t_2 and w_1, w_2 from two to three determinations were used (11).

RIA

Preparation of Antisera

For production of antisera (12) the following peptides were used as antigens: [Leu]enkephalin, [Met]enkephalin, β_h-endorphin(1–16), and β_h-endorphin(27–31). An antiserum, "DA," which was reported (12) to recognize the middle region of β_h-endorphin(1–31), was already available. The antiserum against the β_h-LPH(1–58) segment was purchased from NEN, Dreieich, FRG.

Three micromoles of peptide (molecular weight <1500)

or 2 μmol of peptide (molecular weight >1500) and 25 mg of thyroglobulin were dissolved together in 2.0 ml of distilled water. After incubation for 10 min at room temperature, covalent conjugation was started by adding 1 mg 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide in 1 ml distilled water. The mixture was kept at 4°C overnight, then 3 ml distilled water was added and aliquots were stored at −35°C. For immunization, 1.2 ml of the peptide conjugate was thawed and emulsified with an equal volume of complete Freund's adjuvant.

One milliliter of the conjugate corresponded to 0.17 (molecular weight >1500) or 0.25 (molecular weight <1500) μmol of peptide, respectively. Six-tenths milliliter (rabbit) or 0.2 ml (guinea pig) of the mixture was injected subcutaneously and intradermally at multiple sites in the back of one animal. For immunization against one peptide, three rabbits and three guinea pigs were used. Injections were repeated three times at intervals of 7 days. Two weeks after the last injection, animals were bled and again 2 weeks later boosted with the same amount of antigen as used for primary immunization; instead of complete, incomplete Freund's adjuvant was used. Bleeding and boosting cycles were repeated up to 10 times.

Preparation of Labeled Peptides

The following peptides were labeled with ^{125}I: [Leu]enkephalin, β_h-endorphin(1–16), β_h-endorphin(1–31), β_h-endorphin(27–31), and β_h-LPH(1–89).

Except for β_h-endorphin(1–31), the following radioiodination procedure was employed, using a modification of the chloramine T method of Hunter and Greenwood (13). To a small siliconized glass vial were added, in succcession, 1 mCi of carrier-free Na ^{125}I in 10 μl of a sodium hydroxide solution (Amersham Buchler, Braunschweig, FRG), 10 μl of a 10^{-3} *M* peptide solution (β_h-LPH; 10^{-4} *M*), 50 μl of 0.5 *M* sodium phosphate buffer, pH 7.5, and 20 μg of chloramine T in 10 μl of 0.05 *M* phosphate buffer. After 30 sec the reaction was terminated by the addition of 20 μg of sodium metabisulfite in 10 μl of 0.05 *M* phosphate buffer. All solutions, except the iodine, had been kept on ice until added to the vial for reaction at ambient temperature.

For purification of the tracer, the mixture was immediately applied to a 0.9 × 60-cm Bio-Gel P4 column (Bio-Rad Laboratories, München, FRG) previously equilibrated with 1% acetic acid (v/v) containing 0.1% bovine serum albumin (w/v) and subsequently washed with 1% acetic acid. Elution was performed with 1% acetic acid at a flow rate of approximately 15 ml/hr at 4°C. One hundred 1-ml fractions were collected. Three or four fractions from the ^{125}I-peptide peak were pooled, an equal volume of 1-propanol was added, and aliquots were stored at −35°C. Specific activity was about 200 Ci/mmol.

For radioiodination of β_h-endorphin(1–31) the following method was employed (14): 10 μl of a 0.3×10^{-3} *M* aqueous β_h-endorphin(1–31) solution was added to 100 μl of a 0.25 *M* phosphate buffer, pH 7.4/2 *M* urea mixture. After the addition of 3 mCi carrier-free Na ^{125}I in 30 μl sodium hydroxide solution the reaction was started by adding 10 μg chloramine T in 20 μl H_2O and terminated after 50 sec with 20 μg sodium metabisulfite in 20 μl H_2O. Finally, 100 μl 1% acetic acid was added. Purification of the tracer was performed as described above. The specific activity was about 800 Ci/mmol.

Buffers

Buffers for radioimmunoassays were prepared using a previously described method (15). Buffer 1 consisted of 0.02 *M* sodium phosphate (pH 7.5) containing 0.15 *M* sodium chloride, 0.1% (w/v) gelatin, 0.01% (w/v) bovine serum albumin, and 0.01% (w/v) thimerosal. Buffer 2 consisted of buffer 1 with 0.1% (v/v) Triton X-100 added.

Assay Procedure

Incubations were performed in 1.5-ml Eppendorf micro test tubes (Eppendorf, Hamburg, FRG) on ice.

Standard solutions were prepared using dilutions from synthetic peptides in buffer 2. Antisera were diluted with buffer 1; iodinated peptides, with buffer 2 (ca. 3000 cpm/20 μl buffer).

To 50 μl buffer 1 were added, in succession, 20 μl standard solution ("standard") or 20 μl buffer 2, 100 μl sample ("sample") or 100 μl buffer 1, and 50 μl antiserum solution or 50 μl buffer 1 ("blank"). The tracer (20 μl) was added either immediately or after preincubation for 24 hr. Incubation in the presence of the tracer was performed for 24 hr. Antibody-bound tracer was separated from free tracer by the addition of 300 μl of a charcoal suspension (1 g of charcoal and 0.5 g of bovine serum albumin suspended in 100 ml of buffer 1), incubation for 10 min, and subsequent centrifugation (12,000*g*, 4 min). Four hundred fifty microliters of the supernatant was transferred to another tube and radioactivity therein was counted in a gamma counter (Kontron, Zürich, Switzerland).

RIA standard curves were obtained by displacement of the ^{125}I-labeled peptide from the antibody by the unlabeled corresponding peptide. The IC_{50} and IC_{10} values corresponded to the molar concentrations of unlabeled peptides to inhibit 50 and 10%, respectively, of the binding of the labeled peptide to the antibody. The cross-reactivity (percentage) of any substance tested is defined as 100 times the ratio IC_{50} of the unlabeled peptide/IC_{50} of the tested substance.

The intraassay coefficient of variation for one radioimmunoassay was calculated from determinations of immunoreactivity in 9–11 aliquots from a sample pool; the interassay coefficient of variation was calculated from the IC_{50} values in 10 different radioimmunoassays.

RESULTS

Extraction Method

For blood collection EDTA was chosen as an anticoagulant since heparin was assumed possibly to interfere with β-endorphin extraction from plasma (see Discussion).

Recoveries were determined for [Leu]enkephalin, β_h-endorphin(1–16), β_h-endorphin(1–31), β_h-endorphin(27–31), and β_h-LPH(1–89) (Table I). For the larger peptides, the recovery rates proved to be highly dependent on the processing of the extracts. Small peptides displayed recovery rates obviously independent of the processing of the ex-

Table I. Extraction Method: Peptide Recoveries, Linearity, and Reproducibility Characteristics

	[Leu]Enk	β_h-E(1–16)	β_h-E(1–31)	β_h-E(27–31)	β_h-LPH(1–89)
Recovery ($\bar{X}$ %)					
Unlabeled peptides[a–c]	86	64	48	8	21
Unlabeled peptides[c–e]	81	87	90	7	34
Labeled peptides[c,e,f]	91	100	95	8	43
Linearity					
Correlation coefficient[b]	0.99	0.98	0.98	0.93	0.92
Intraassay coefficient of variation (%)[b]	6.2	8.0	7.2	10.8	12.3
Interassay coefficient of variation (%)[b]	4.7	9.2	7.1	14.6	8.0

[a] Extracts reconstituted in buffer 1 for radioimmunoassay.
[b] Number of experiments $N = 10$.
[c] Mean values.
[d] Extracts processed as described for HPLC samples (see Materials and Methods), dried, and reconstituted in buffer 1 for radioimmunoassay.
[e] Number of experiments $N = 2–3$.
[f] Radioactivity determined directly in the cartridge eluate.

tracts; however, their absolute recovery values proved to be highly different.

The highest recovery rates were found for the radiolabeled peptides; the radioactivity had been determined directly in the cartridge eluate, whereas the peptide immunoreactivities had been determined in extracts reconstituted following different protocols.

Recovery rates seemed to be independent of the amount of peptide extracted within a range from 2 to 500 fmol/ml plasma supernatant. Reproducibility characteristics in the form of intra- and interassay coefficients of variation are also given in Table I.

HPLC

HPLC characteristics are given in Table II. In the table the retention times of extracted peptides are given; peptides chromatographed directly showed slightly prolonged retention times of about 1 min.

As a measure for efficiency of peptide separation, resolution values R_s (see Materials and Methods) are given for extracted peptides. The values for directly chromatographed peptides were nearly identical.

It must be emphasized that by admixture of amino acids to the organic modifier even an effective resolution of β_h-endorphin and β_h-LPH was achieved.

The sample volume injected varied from 100 to 1800 μl, which did not significantly influence retention times and resolution. The recovery of the peptides proved to be correlated with the molecular size of the peptides.

RIA

A multiple radioimmunoassay system was developed for determination of β-endorphin and fragments thereof in

Table II. HPLC: Peptide Recoveries and Efficiency of Peptide Separation

	β_h-E(27–31)	[Leu]Enk	[Met]Enk	β_h-E(1–16)	β_h-E(1–31)	β_h-LPH(1–89)
Retention time (min)[a–c]	4.5	26.5	17.5	23.0	31.0	27.5
Resolution[a,b,d]						
β_h-E(27–31)						
[Leu]Enk	10.2					
[Met]Enk	6.8	4.2				
β_h-E(1–16)	8.9	1.2	2.9			
β_h-E(1–31)	10.8	1.5	3.4	2.5		
β_h-LPH(1–89)	9.6	0.4	4.2	1.5	1.0	
Recovery (%)[a–c]	68	89	78	50	31	22

[a] Extracted peptides.
[b] Number of experiments $N = 2–3$.
[c] Mean values.
[d] Resolution values for pairs of peptides as listed.

extracts of human plasma. Optimum conditions for an RIA using antiserum 5 (see Table III) were established and the other radioimmunoassays were performed using the established protocol.

Separation of free from antibody-bound ^{125}I-β_h-endorphin was tested using a charcoal suspension (see Materials and Methods), a charcoal–dextran mixture, or talcum as the adsorbent as well as double-antibody immunopreciptation. Maximum sensitivity of the assay could be achieved likewise by separation using the charcoal suspension or by immunoprecipitation. Because of easier and faster processing the charcoal separation technique was chosen for routine assay.

Radioimmunoassay 5 was optimized systematically by testing the influence of pH, ions, detergents, denaturing agents, and antioxidants. At a pH of 7.5, the best reproduci-

Table III. Radioimmunoassays: Antisera, Sensitivity, Specificity, and Reproducibility Characteristics

	Antiserum					
	1	2	3	4	5	6
Antigen[a]	[Leu]Enk	β_h-E(1–16)	β_h-E(1–16)	β_h-E(27–31)		
Final dilution of antiserum	1:19,200	1:9600	1:9600	1:3400	1:96,000	Diluted according to prescription
IC_{10} (fmol/assay tube)[b]						
RIA: immediate tracer addition[c]	2.4	8.9	1.3	2.7	4.2	2.3
RIA: delayed tracer addition[d]	0.7		0.8	1.4	0.9	
IC_{50} (fmol/assay tube)[b]						
RIA: immediate tracer addition[c]	55	52	11	24	35	31
RIA: delayed tracer addition[d]	25		9.5	10	8.3	
Cross-reactivity (%)[e]						
[Leu]Enk	100	<0.01	<0.01	<0.01	<0.01	0
[Met]Enk	20	<0.01	<0.01	<0.01	<0.01	0
β_h-E(1–9)	<0.01	<0.01	15	<0.01	<0.01	
β_h-E(1–16)	<0.01	100	100	<0.01	<0.01	
β_h-E(1–31)	<0.01	<1	59	41	100	0
β_h-E(27–31)	<0.01	<0.01	<0.01	100	<0.01	
β_h-LPH(1–89)	<0.01	<0.1	10	24	54	100
β_c-E(1–31)				0.1		
Lys–Lys				<0.01		
Lys–Gly–Glu				0.1		
Tyr–Lys				<0.01		
β-Endorphin segment binding to the antibody[f]	|(1–5)|[g]	←(9–16)|[g]	←(5–16)→[h]	←(27–31)|[h]	←(17–26)→[h]	β_h-LPH(1–58) segment interacting with antibody
Intraassay coefficient of variation (%)[i]	2.3		5.8	6.0	6.3	6.8
Interassay coefficient of variation (%)[i]	9.5		4.0	7.2	6.8	10.4

[a] Used for generation of antibody.

[b] Amount of unlabeled peptide required to inhibit binding of the ^{125}I-labeled peptide to the antibody by 50 or 10%, respectively.

[c] RIA incubation period: tracer added immediately.

[d] RIA incubation period: tracer added after preincubation for 24 hr.

[e] Calculated as 100 times the ratio IC_{50} of the unlabeled peptide corresponding to the ^{125}I-labeled peptide/IC_{50} of the tested substance. RIA 6: information on cross-reactivities from NEN.

[f] Numbers, β_h-endorphin amino acid sequence positions limiting the segment which apparently interacted with the respective antibody ("binding segment"); ←/→, elongation of binding segment into arrow's direction—interaction of the respective antibody with an elongated binding segment was observed; |, interaction of the respective antibody with an elongated binding segment was not observed.

[g] "Binding segment" not recognized by the antibody when incorporated in the β_h-endorphin (1–31) molecule.

[h] "Binding segment" recognized by the antibody, whether incorporated in the β_h-endorphin (1–31) molecule or occurring as an isolated fragment.

[i] Number of experiments N = 9–11.

bility of the results as well as a low intraassay variation was obtained. Because 0.05 *M* phosphate affected antibody–antigen binding, a concentration of 0.02 *M* phosphate was adopted for standard assay. Urea (1 *M*), ascorbic acid (1%; w/v), and mercaptoethanol (1%; v/v) interfered with antibody–antigen binding. Triton X-100 had no influence on antigen–antibody interaction up to a concentration of 0.14% (v/v).

In Table III the characteristics of the established radioimmunoassays are given. Antisera were obtained using [Leu]enkephalin, β_h-endorphin(1–16), and β_h-endorphin(27–31) as antigens. In addition, an antiserum against the middle region of β_h-endorphin(1–31) as well as commercially available antiserum against β_h-LPH(1–58) was employed. Radioimmunoassays 1–6 were developed using these antisera.

Final antiserum dilutions in the assay tube were between 1:3400 and 1:96,000 to achieve tracer binding between 25 and 35%. The degradation of the tracers during RIA incubation was tested according to Gay and Lahti (16). No evidence for degradation was found.

Standard curves were obtained by displacement of the ^{125}I-labeled peptide by the corresponding unlabeled peptide; IC_{10} or IC_{50} values as listed represent the amounts of the unlabeled peptide required to inhibit binding of the ^{125}I-labeled peptide to the antibody by 10 or 50%, respectively. In view of validity criteria (linearity of standard curve, low variance), the IC_{10} values were regarded as the minimum detectable amounts of peptides in the assay tube. The minimum detectable amounts were decreased, i.e., the sensitivity of the assay was increased considerably, when the tracer was added following preincubation for 24 hr (see Materials and Methods).

The cross-reactivities of β_h-endorphin, β_h-endorphin fragments, or further peptides with the antisera listed in Table III indicate the degree of specificity of the various radioimmunoassays. From these cross-reactivity values, the β_h-endorphin segments recognized by the various antibodies used were derived.

Reproducibility characteristics in the form of intra- and interassay coefficients of variation are also given in Table III.

β_h-Endorphin or Fragments Thereof in Plasma of Volunteers Under "Normal" Conditions

The described method was used to determine β_h-endorphin or fragments thereof in the plasma of humans; the blood samples were drawn under "nonstress" conditions.

Two hundred milliliters of plasma from a healthy young volunteer was extracted, the extract was chromatographed, and the HPLC column eluates were assayed using antisera 3, 4, and 5. These antisera recognize (see Table III) β_h-endorphin segments 6–16, 17–26, and 27–31. Thus, an intact β_h-endorphin(1–31) molecule should be recognized by all three antisera. In addition, RIA 6 was also used; the respective antiserum recognizes an unknown segment of the β_h-LPH(1–58) sequence. An intact β_h-LPH(1–89) molecule should be recognized by antisera 3, 4, 5, and 6.

Figure 1 (left) shows the results. Strikingly, an immunoreactive material eluted at the β_h-endorphin(1–31) position was recognized only by antisera 3 and 4, which means that a

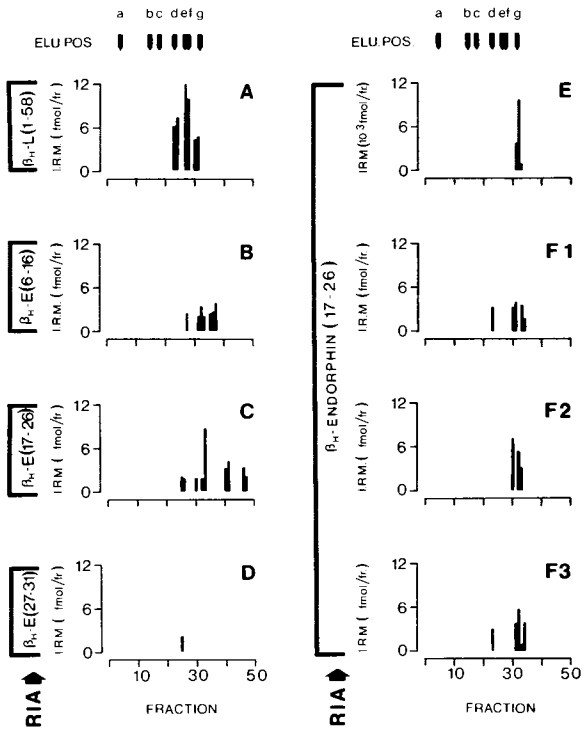

Fig. 1. Analysis of extracts from plasma obtained from volunteers under "nonstress" conditions by HPLC and multiple RIA of eluates. Left: Chromatography of an extract from plasma (200 ml) from one single donor; the eluate was analyzed by radioimmunoassays for β_h-LPH(1–58) (A) and for segments of the β_h-endorphin molecule as indicated (B, C, D). Right: Chromatography of extracted synthetic β_h-endorphin (E) and chromatography of extracts of plasma obtained from three donors (100 ml plasma each) (F1, F2, F3); the eluates in E, F1, F2, and F3 were analyzed by a radioimmunoassay for a middle region of β_h-endorphin. Elution positions of extracted standards are indicated at the top: a, β_h-endorphin(27–31); b, β_h-endorphin(1–9); c, [Met]enkephalin; d, β_h-endorphin(1–16); e, [Leu]enkephalin; f, β_h-LPH(1–89); g, β_h-endorphin(1–31). I.R.M., immunoreactive material; fmol/fr., fmol/fraction; β_h-L, human β-lipotropin.

β_h-endorphin fragment eluted from the column whose C terminus was obviously not identical to the C-terminal fragment of β_h-endorphin(1–31). Since modification of β_h-endorphin(1–31) due to extraction or HPLC procedures can be excluded [when β_h-endorphin was subjected to extraction and HPLC procedures as described, a material was found at the β_h-endorphin(1–31) elution position which was recognized by all three antisera], the volunteer's plasma must be assumed to contain no β_h-endorphin(1–31).

The immunoreactive material eluted at the β_h-LPH(1–89) elution position was not recognized by antisera 4 and 5; thus, the β_h-LPH(1–89) molecule also was not present in the volunteer's plasma. Instead, several β_h-endorphin fragments showed up in the eluates (Fig. 1, left); none of them could be identified by comparison with elution positions and immunoreactive properties of reference peptides.

In view of the results obtained with one single donor, it seemed to be important to examine whether interindividual differences between β_h-endorphin immunoreactive materials in the plasma of humans under nonstress conditions might occur. Therefore plasma samples from three male healthy

volunteers were extracted (100 ml each), the extracts chromatographed, and the column eluates assayed using RIA 5; the respective antiserum recognizes the β_h-endorphin segment 17–26.

The result is shown in Fig. 1 (F1, F2, and F3). As with donor 1 (see Fig. 1, left) an immunoreactive material eluting at the β_h-endorphin(1–31) position was recognized by the antiserum in all three eluates. In none of the eluates did an immunoreactive material appear at the β_h-LPH(1–89) elution position. Instead, other immunoreactive materials appeared—however, these materials were not found in each of the eluates.

Nevertheless, total plasma concentrations of β_h-endorphin immunoreactive materials were calculated to be in the range described by other investigators. The extraction of synthetic β_h-endorphin resulted in one single peak (Fig. 1, E), demonstrating that the diversity of the β_h-endorphin(17–26) immunoreactive peptides (antiserum 5) as found was not due to peptide degradation during extraction or HPLC processing.

DISCUSSION

A method for the determination of β_h-endorphin and fragments thereof in human plasma was developed. The method includes extraction of peptides using octadecasilyl-silica cartridges, reversed-phase HPLC, and a multiple radioimmunoassay system.

For blood collection the choice of the anticoagulant is of importance. Recent studies (17) have shown that heparin can induce the formation of high-affinity binding sites for β_h-endorphin in human plasma; in contrast, EDTA induces only minor amounts of binding sites. Therefore EDTA was chosen as the anticoagulant.

Extraction of β_h-endorphin immunoreactive materials has been widely performed using silica gel as an adsorbent (2,8,18). In our hands, studies with different batches of silica gel indicated that extracts may contain contaminants affecting antibody–ligand interactions in the β_h-endorphin RIA. Therefore, silica gel was replaced by octadecasilyl-silica (cartridges) (9,19). In fact, these extracts proved to be free of contaminants interfering with the RIA; since the β_h-endorphin recovery rates (extract reconstitution directly in RIA buffer) proved to be almost identical for the two methods, an octadecasilyl-silica cartridge extraction method was established and also used for the other peptides to be extracted.

For larger peptides (in their unlabeled form), the recovery rates proved lower when the extracts were reconstituted in RIA buffer than for extracts processed according to the HPLC protocol. However, for those effects, the type of solvent used for reconstitution obviously was not responsible, since dissolving of the extracts in HPLC solvent B, subsequent drying, and reconstitution in RIA buffer did not improve the recovery rate. Better recovery was achieved only by dissolving the extracts in HPLC solvent B and further processing (two filtration steps) as described for HPLC sample preparation. This phenomenon might be due to an adsorption of peptides when the extracts were reconstituted in RIA buffer either to particles originating from the packing material of the cartridge or to precipitates from the sample itself. In contrast, in solvent B, this adsorption effect obviously did not occur and the peptide-adsorbing particles were presumably retained by the filtration process. Recovery rates obtained with "filtered" extracts corresponded to recovery rates determined with iodinated peptides and are, in part, in agreement with data from other investigators (9).

To achieve an efficient separation of β_h-LPH and the opioid peptides under study, a number of chromatography systems were tested. Although conventional gel filtration on Sephadex G75 (Pharmacia, Uppsala, Sweden) resulted in a satisfactory separation of β_h-endorphin and β_h-LPH as reported before (20), only poor resolution for the other peptides was observed. Thus, separation by HPLC was tried, but a method which promised to be appropriate for separation of all peptides (21) was not employed, since at a temperature of 45°C as recommended, decomposition might have occurred (22). Instead, other systems were tested. Several approaches including isocratic or gradient elution using μ-Porasil, I-60, or μ-Bondapak HPLC columns (Waters Associates, Bedford, Mass.) were successful as far as the separation of the smaller peptides was concerned; however, a separation of β_h-LPH from β_h-endorphin could not be achieved. Only the addition of amino acids to the organic modifier of the employed system led to a sufficient extension of the difference between the retention time of β_h-LPH and that of β_h-endorphin.

A severe disadvantage in reversed-phase HPLC is a low recovery rate especially for larger peptides in the low femtomole range (22,23). Presumably, this is due to an irreversible adsorption of the peptides to the packing material of the column (22) rather than to a problem with reconstitution of the eluates. In our hands, the addition of glycylclycine to the HPLC solvents did not improve the recovery rates (23); however, the addition of amino acids to the organic modifier improved not only the resolution but also the recovery rates of the peptides.

Frequently, an antibody is generated using a certain peptide as antigen, and whenever an immunoreactive material can be demonstrated in a radioimmunoassay established using that antibody, the material, more or less tacitly, is assumed to be identical to the antigen. However, the antibody usually is just able to recognize a certain segment of the peptide, and thus, the immunoreactive material may contain this segment, but the rest of its amino acid sequence may be entirely different from that of the antigen. Therefore, for determination of β_h-endorphin (and also separate fragments thereof) a multiple RIA system was developed to identify the β_h-endorphin sequence, as far as possible, "piece by piece"; the development of a method for determination of the N terminus of β_h-endorphin (24), so far lacking, is in progress. Although most of the radioimmunoassays developed for determination of β_h-endorphin were based on one antibody only (1–9), there have been, in fact, sporadic efforts to establish radioimmunoassay systems using more than one antibody (12,25); however, they were not designed to establish this kind of radioimmunological structure identification method.

Separate determination of the β_h-endorphin segments, in particular, deserves interest in view of the different functional significance of various parts of the β_h-endorphin se-

quence: whereas the N terminus is known to interact with opioid receptors, the C terminus appears to be important for an immunological function of β_h-endorphin (26).

In fact, using the developed method, it was possible to demonstrate that in volunteers' plasma drawn under "non-stress" conditions β_h-endorphin immunoreactive materials may occur which are identical neither to β_h-endorphin nor to β_h-LPH. This finding confirms preliminary results (27) and has not been raised by others in such detail so far.

Other groups (1,2,6,8,9) searching for β_h-endorphin in "normal" human plasma had reported β_h-endorphin immunoreactive materials eluted at chromatographical β_h-endorphin elution positions. Although only one antibody against β_h-endorphin had been used, the respective materials had been assumed to represent intact β_h-endorphin. In this study, β_h-endorphin immunoreactive materials were found at the β_h-endorphin HPLC elution positions and, using a multiple RIA, evidence was presented that these materials were not identical with intact β_h-endorphin.

However, it should be emphasized that the β_h-endorphin-related peptides found in the plasma of nonstressed volunteers do not necessarily reflect peptides as released from the pituitary but might represent products from β_h-endorphin breakdown or other modification occurring after release, as well.

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