A sensitive and specific double-antibody enzyme immunoassay (EIA) for nociceptin (orphanin FQ)-like immunoreactive substances (nociceptin-IS) was developed. In competitive reactions, the nociceptin-antibody was incubated with both a nociceptin standard (or plasma extract sample) and β-D-galactosidase (β-Gal)-labeled synthetic human nociceptin (delayed addition method). The free and antibody-bound enzyme haptens were separated using an anti-rabbit IgG-coated immunoplate. The enzyme activity on the immunoplate was determined fluorometrically. The present immunoassay allows the detection of 15–700 pg/ml (sensitivity: 1.5 pg, 0.6 pg/well) of nociceptin. Using this EIA, the nociceptin-IS levels in human plasma were determined, and found to be in the range of 5.0 to 16.0 pg/ml. No circadian rhythms in the daytime (9:00–19:00) or effects of eating meals on human plasma nociceptin levels were found. We have established an evaluation system for both nociceptin-IS and substance P-IS levels in 1 ml of human plasma. As for the evaluation of analgesic effects of drugs and pain, this sensitive and specific EIA system for endogenous nociceptin may be valuable for clinical use.

Key words —— nociceptin (orphanin FQ), enzyme immunoassay, human plasma, substance P

INTRODUCTION

Nociceptin/orphanin FQ (a 17 amino-acid peptide) is the endogenous ligand for the Opioid Receptor-Like 1 (ORL1) receptor.1,2) This neuropeptide is derived from the precursor prepronociceptin gene, which is expressed in the central nervous system and peripheral tissues.3) Nociceptin has been shown to exert a variety of biological actions, some of which are opposite to those evoked by classical opioids.4,5) Nociceptin has been implicated in the regulation of a variety of behaviors and physiological processes. It has been most extensively studied in relation to its influence on nociceptive responsiveness.6–8) Recently, nociceptin has been reported to affect stimulation of food consumption,9) cardiovascular control,10) locomotor activities,11) learning and memory processes,12,13) water electrolyte balances,14) etc.

Recently, searching for a pure and selective antagonist for nociceptin receptors (ORL1), and regulation of endogenous nociceptin as a nociceptive mediator in the humoral system, have been one of the new strategies for controlling pain and physiological processes in man.15,16) As for the evaluation of the analgesic effects of drugs and pain, sensitive and specific measurement of nociceptin in the humoral system might be valuable and necessary for clinical use.

Radio immunoassays (RIA) for detecting nociceptin have been developed by a few groups using radioisotope-labeled nociceptin.17–19) However, in terms of safety, sensitivity, and ease of handling, RIA methods are still less than satisfactory. We developed a sensitive and specific double-antibody enzyme immunoassay (EIA) for detecting nociceptin, using nociceptin-linked β-D-galactosidase (β-Gal) as a marker antigen, a secondary antibody-coated immunoplate and 4-methylumbelliferyl-β-D-galactopyranoside as a fluorogenic substrate, and applied this proposed EIA to the measurement of nociceptin in human plasma for clinical use.
The purposes of this study were to detail the sensitive and specific EIA for detecting nociceptin, and to examine the existence of circadian tendency of nociceptin in the daytime on human plasma for practical use.

**MATERIALS AND METHODS**

**Materials** —— Synthetic human nociceptin, substance P, dynorphin A, β-endorphin, adrenocorticotrophic hormone (ACTH) (1–24), vasoactive intestinal peptide (VIP), somatostatin, and α-atrial natriuretic peptide (α-ANP) were purchased from Peptide Institute Inc. (Osaka, Japan). Nociceptin (1–13) amide was purchased from Bachem AG (Bubendorf, Switzerland). Antisera to nociceptin (AB5114) and substance P (RA-08-095) were purchased from CHEMICON International (Temecula, CA, U.S.A.) and Cambridge Research Biochemicals (Cambridge, U.K.), respectively. Goat affinity-purified antibody to rabbit IgG (whole molecule) (55641) was purchased from Sigma (St. Louis, MO, U.S.A.). 4-Methylumbelliferyl-β-D-galactopyranoside (MUG) and N-ε-maleimidocaproyloxy (EMC)-succinimide were purchased from Sigma (St. Louis, MO, U.S.A.). β-Gal and aprotinin (Trasylol®) were purchased from Boehringer Mannheim (Mannheim, Germany) and Bayer (Leverkusen, Germany), respectively. All other reagents were commercially available and of reagent grade.

**Preparation of Enzyme-Labeled Antigens** —— Human nociceptin and substance P were conjugated with β-Gal by EMC-succinimide. These peptides dissolved in 0.05 M phosphate buffer (pH 7.0) were mixed with EMC-succinimide in tetrahydrofuran at 35°C overnight, washed with 0.01 M β-Gal and aprotinin (Trasylol®), and then added, and the solution was incubated at 4°C for an additional 24 hr. 100 µl of the antigen-antibody solution for each sample was added to the secondary antibody-coated immunoplate. The plate was incubated at 4°C overnight, washed with 0.01 M phosphate buffer (pH 7.0) containing 0.15 M NaCl, and 0.05% Tween 20, and then 200 µl of 0.1 mM MUG in 0.05 M phosphate buffer (pH 7.0) containing 1 mM MgCl₂ was added to each well. The plate was incubated at 37°C for 180 min, and then the fluorescence intensity (λ Ex 360 nm, λ Em 450 nm) of the fluorescent product, 4-methylumbelliferon, was measured with an MTP-100F microplate reader (Corona Electric, Ibaraki, Japan).

**Preparation of Plasma Extracts** —— The blood samples were placed in chilled tubes containing aprotinin (500 KIU/ml) and ethylenediaminetetraacetic acid (EDTA) (1.2 mg/ml). After centrifugation, the plasma was diluted with 4% acetic acid (pH 4.0), loaded onto Sep-Pak® C₁₈ cartridges (Millipore, MA, U.S.A.), and washed with 4% acetic acid. The peptides in the plasma were eluted with 70% acetonitrile in 0.5% acetic acid (pH 4.0), lyophilized, reconstituted to 100 µl with the assay buffer, and subjected to ELA. For the nociceptin EIA system, plasma samples were concentrated five-fold with Sep-Pak® C₁₈ cartridges. The recovery and reproducibility for human plasma with this nociceptin EIA were examined by adding standard solution to hormone-free plasma.

**HPLC of Plasma Extracts** —— HPLC was performed using a reverse phase C₁₈ packed column (Cosmosil 5C₁₈-AR, Nacalai Tesque, Kyoto, Japan). The HPLC consisted of a model 600E pump system (Millipore). The plasma samples (2.5 ml), purified by Sep-Pak® C₁₈ cartridges as described above, were reconstituted in the first composed mobile phase and passed through the column. Nociceptin-IS was eluted with a linear gradient of acetonitrile (from 5% to 50% over 45 min) in 0.1% trifluoroacetic acid. The flow rate was 1.0 ml/min and the fraction size was 1.0 ml. Eluted fractions were concentrated by spin-vacuum evaporation, lyophilized, and stored (–40°C)
Subjects —– Five healthy male volunteers (non-smokers), aged 25–28 (median, 26 years), participated in this study. Each subject received information about the study’s scientific purpose, which was approved by the Ethics Committee at Oita Medical University, and gave informed consent. No subject received any medication for a month preceding the test.

Study Schedule —– Venous blood samples from a forearm vein were taken to measure the levels of each peptide in the plasma by EIA. Blood samples were taken at 9:00, 11:00, 13:00, 15:00, 17:00, and 19:00. All subjects ate breakfast at 6:45–7:15 and lunch at 11:45–12:15, and the volunteers did not take food or drink outside the above periods.

Data Analysis —– All values were expressed as the means ± S.D. Comparisons of plasma peptide levels among blood sampling times were made by one-way analysis of variance. A value of $p < 0.05$ was regarded as significant.

RESULTS

Standard Curve
A typical calibration curve for the nociceptin EIA is shown in Fig. 1. When plotted as a semi-logarithmic function, a linear displacement of enzyme-linked nociceptin by nociceptin was noted between 15 and 700 pg/ml with antiserum AB5114. The minimum amount of nociceptin detectable by these EIA systems was 1.5 pg (0.6 pg/well) with antiserum AB5114, and the IC$_{50}$ of the calibration curve was 80 pg/ml.

Specificity of Antiserum AB5114
The immunospecificity of antiserum AB5114 was examined by EIA using nociceptin conjugated with β-Gal. The displacement curves (1, 10, 100, 1000, 10000, 100000 pg/ml) of nociceptin (1–13) amide, and other endogenous peptides [human substance P, dynorphin A, β-endorphin, ACTH (1–24), VIP, somatostatin, α-ANP] are shown in Fig. 1. Substance P, dynorphin A, β-endorphin, ACTH (1–24), VIP, somatostatin, and α-ANP showed minimal inhibition of the binding of β-Gal-conjugated nociceptin with nociceptin antiserum AB5114 (> 88%). The amino-terminal nociceptin fragment (1–13) amide exhibited little cross-reactivity. Thus, nociceptin antiserum AB5114, which recognizes nociceptin outside of fragment (1–13), can distinguish nociceptin from other related neuropeptides (substance P, dynorphin A, β-endorphin, etc.).

Measurements of Nociceptin-Like Immunoreactive Substances in Human Plasma by EIA
Human plasma extracts were subjected to reverse-phase HPLC to assess the presence of nociceptin-IS molecular variants in human plasma. The elution profiles revealed the presence of main immunoreactive peak (arrow), eluting at a position corresponding to standard nociceptin, and several unknown peaks (Fig. 2). The unknown immunoreactive peaks were found at fraction number 10, 25, and 40 around. The recovery rates of human plasma nociceptin between the proposed detectable range (15 pg/ml and 700 pg/ml) with this EIA were 94.3% and 96.8%, respectively. The reproducibility (CV%) for human plasma (15 pg/ml and 700 pg/ml) with this nociceptin EIA was 4.9% and 3.8% for intra-assay ($n = 10$) comparisons.

Circadian Rhythms in the Daytime of Nociceptin-IS and Substance P-IS in Human Plasma
The circadian rhythms of the human plasma nociceptin-IS levels in the daytime are shown in Fig. 3. The mean nociceptin-IS levels at each time point are 8.4 ± 3.4 pg/ml at 9:00, 12.2 ± 2.6 pg/ml at 11:00, 7.9 ± 3.1 pg/ml at 13:00, 11.2 ± 3.3 pg/ml at 15:00, 9.7 ± 4.1 pg/ml at 17:00, and 8.3 ± 3.8 pg/ml at 19:00, and these values showed a range of 5.0 pg/ml to 16.0 pg/ml. The nociceptin-IS levels
among these daytime points (9:00, 11:00, 13:00, 15:00, 17:00, and 19:00) showed no significant differences ($p = 0.291$).

The circadian rhythms of human plasma substance P-IS levels in the daytime are shown in Fig. 4. Plasma substance P levels showed a range of 9.4 pg/ml to 34.0 pg/ml. Substance P-IS levels among the daytime points (9:00, 11:00, 13:00, 15:00, 17:00, and 19:00) showed no significant differences ($p = 0.098$).

**DISCUSSION**

Using β-Gal-labeled nociceptin as a marker antigen, an anti-rabbit IgG-coated immunoplate as a bound/free separator, and MUG as a fluorogenic substrate, we have developed a sensitive and specific EIA for the quantification of nociceptin in human plasma. Since 1997, a few RIA methods developed for nociceptin have been used in human and animal studies. However, these methods have several disadvantages due to the use of radioisotopes. The EIA detailed in this report retains the advantages of the RIA system, while minimizing the disadvantages.

This EIA is sensitive (1.5 pg, 0.6 pg/well) and specific (carboxy-terminal region) for nociceptin, and the sharp standard inhibition curve obtained was linear between 15 and 700 pg/ml. The sensitivities of RIA have previously been reported as 1.56 pg (12 pg/ml), 17] 3.3 pg (1.8 fmol), 18] and 995 pg (0.55 pmol). 19] Recently, an EIA system for nociceptin (EK-021-55, Phoenix Pharmaceuticals Inc., CA, U.S.A.) was placed on the market, but the minimum detectable concentration of this EIA is 280 pg/ml, and the sensitivity is 14 pg. With regard to the operation, our EIA enables the measurement of many samples (96 wells) at the same time by using an anti-rabbit IgG-coated immunoplate as the bound/free separator. The nociceptin antibody AB5114 was found to have no cross-reactivity with the structurally related peptide, dynorphin A, and this assay system is valuable and necessary for evaluation with opioid peptides, dynorphin A and β-endorphin, and pain-related peptide, substance P, at the same time.

There have been few reports that developed an EIA for nociceptin and applied this EIA to human plasma. We applied our EIA to the detection of plasma nociceptin-IS levels. The IC$_{50}$ of this nociceptin EIA was 80 pg/ml, and thus plasma samples had to be concentrated 5-fold with Sep-Pak®
C_{18} cartridges and 500 µl was needed. The human plasma levels in our EIA from 5.0 pg/ml to 16.0 pg/ml in the daytime were nearly equivalent to those obtained with other RIA methods. The detectable plasma levels by RIA reported previously were 7.59 pg/ml (mean) and 7–13 pg/ml. The recovery (> 94%) and reproducibility (CV% of inner-assay and intra-assay comparisons) of this EIA with the plasma samples were satisfactory.

The molecular heterogeneity in human plasma was examined by HPLC. The main nociceptin-IS in plasma was eluted at the same elution time as synthetic human nociceptin with several unknown peaks. This elution profiles resemble HPLC elution profile of rat dorsal spinal cord. These unknown peaks might be fragments of nociceptin, containing the carboxy-terminal region (14–17), since nociceptin has four critical enzymatic cleavage sites, and five main fragments (1–7), (1–11), (2–17), (12–17), and (13–17). Nociceptin is metabolized by aminopeptidase N and endopeptidase 24.15. And nociceptive effect of fragment (13–17), formed after enzymatic cleavage, could be expected to exert some physiological and pharmacological actions. Our proposed nociceptin-EIA recognized carboxy-terminal region specifically, and might be useful for clinical evaluation.

The circadian rhythms of nociceptin in the daytime were investigated for application to clinical use in human plasma. We could not find significant circadian rhythms for human plasma nociceptin levels among the daytime time points (9:00–19:00). Furthermore, no effects of eating meals (breakfast and lunch) on nociceptin levels in human plasma were found. Circadian rhythms and effects of eating meals did not exist for substance P either, and the levels of substance P had no correlation to those of nociceptin in the plasma. Thus, this nociceptin EIA system might be valuable for clinical evaluation in the daytime. Furthermore, using our nociceptin EIA system, it is possible to measure both peptides (nociceptin and substance P) from the same 1-ml of human plasma after concentration with a Sep-Pak{superscript}® C_{18} cartridge.

There have been a few reports that examined clinical evaluation for pain with plasma nociceptin levels in humans. In a practical clinical study, human cerebrospinal fluid and plasma nociceptin levels were investigated in pain and non-pain states. Furthermore, changes in the blood nociceptin levels were analyzed quantitatively in patients with acute and chronic pain, and the nociceptin levels had a relationship with the existence of pain and its duration.

We have established a sensitive and specific EIA system for endogenous nociceptin in human plasma, which will be valuable for application to the clinical evaluation of pain and analgesic effects of drugs, etc.

REFERENCES


