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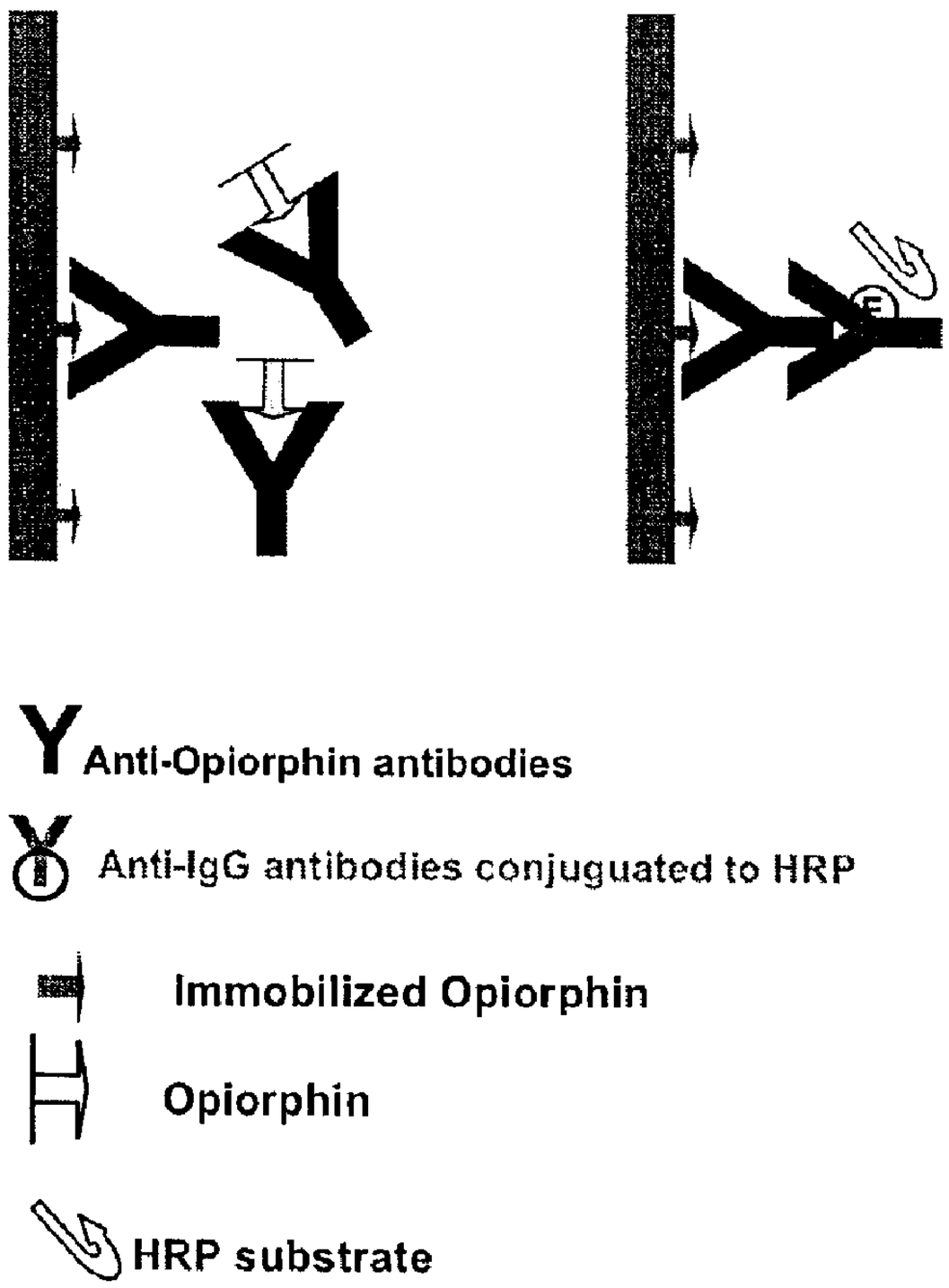
(54) Title: USE OF BASIC PROLIN-RICH LACRIMAL GENE PRODUCTS, SUCH AS OPIORPHIN, AS A BIOMARKER



(57) Abrégé/Abstract:
The present invention relates to the use of Basic Prolin-rich Lacrimal protein (BPLP) gene products, such as Opiorphin, for establishing a prognosis, a diagnosis or the monitoring of a pathological state in a subject and the related method of use.

ABSTRACT

The present invention relates to the use of Basic Prolin-rich Lacrimal protein (BPLP) gene products, such as Opiorphin, for establishing a prognosis, a diagnosis or the monitoring of a pathological state in a subject and the related method of use.

USE OF BASIC PROLIN-RICH LACRIMAL GENE PRODUCTS, SUCH AS OPIORPHIN, AS A BIOMARKER.

FIELD OF THE INVENTION

The present invention relates to Basic Prolin-rich Lacrimal Protein (BPLP) gene products, such as Opiorphin, and more particularly to the use of BPLP gene products, such as Opiorphin, as a biomarker for prognosis, diagnosis or monitoring of a pathological state in a subject and the related method of use.

BRIEF DESCRIPTION OF THE PRIOR ART

The convergent data obtained from integrative post-genomic, biochemistry and pharmacological approaches provide evidence for the existence in mammals of a physiological antagonist of NEP (Neutral EndoPeptidase, Neprilysin) and AP-N (Aminopeptidase-N) ecto-enkephalinases. The inhibitors were characterized firstly in rat (QHNPR-peptide) and named Sialorphin (as described in WO 90/03981, WO 98/00956, WO 01/00221, WO 02/041434), and more recently in Human (QRFSR-peptide) and called Opiorphin (as described in WO 05/090386).

Sialorphin is an exocrine and endocrine peptide-signal. It is an inhibitor of pain perception and acts by potentiating endogenous μ- and δ-opioid receptor-dependent enkephalinergic pathways. Its expression is under activational androgenic regulation and its secretion is evoked under adrenergic-mediated response to environmental stress in male rat (Rougeot et al., 1997, Am. J.Physiol. 273 (4pt2), R1309-1320).

Sialorphin recognizes specific target sites in organs that are deeply involved in the mineral ion concentration and thus may have a therapeutic role in the metabolic

disorders related to a mineral ion imbalance, such as bone, teeth, renal, kidney intestine, pancreas, stomach mucosa or parathyroid disorders caused principally by a mineral ion imbalance in the body fluids or tissues. Accordingly, Sialorphin may be used for preventing or treating diseases like hyper- or hypo-parathyroidism, osteoporosis, pancreatitis, submandibular gland lithiasis, nephrolithiasis or osteodystrophy (WO 98/37100).

Sialorphin may also be used for the treatment of DSM-III disorders (see Diagnostic and Statistical Manual of Mental Disorders, American Psychiatric Assoc. 1992), such as impaired interpersonal and behavioral disorders, including sexual disorders such as male erectile dysfunction (M.E.D.) and hypoactive sexual desire disorder (H.S.D.D.), by providing improved awareness and alertness to environment, improved adaptation to environment and ability to sustain attention, and increased interest in environment and capacity for arousal, without increased aggressiveness (WO 01/00221).

Opiorphin is considered the functional human homologous of Sialorphin. It derives from the BPLP protein ("Basic Prolin-rich Lacrimal Protein") (WO 05/090386, Rougeot et al.). The gene BPLP is expressed in human lacrimal and submandibular glands.

Opiorphin is a QRFSR peptide that inhibits two enkephalin-catabolizing ectoenzymes, human neutral ecto-endopeptidase, hNEP (EC 3.4.24.11), and human ecto-aminopeptidase, hAP-N (EC 3.4.11.2). Opiorphin displays potent analgesic activity in chemical and mechanical pain models by activating endogenous opioid-dependent transmission. The pain-suppressive potency of Opiorphin is as effective as morphine in the behavioral rat model of acute mechanical pain, the pin-pain test. Opiorphin is involved in endogenous opioid-dependent pathways, notably in modulating mood-related states and pain sensation. Furthermore, because of its *in vivo* properties, Opiorphin may have therapeutic implications as a potential initiator of molecular

pathways that could be exploited to develop new candidate drugs for the clinical management of pain relief and the alleviation of emotional disorders (Wisner et al., PNAS, 103 (47): 17979-17984 (2006)). Opiorphin may also be used for the prevention or treatment of any hydro-mineral imbalance, including disorders such as bone, teeth, kidney, parathyroid, pancreas, intestine, stomachmucosa, and salivary gland disorders. The disorder may be hyper- or hypo-parathyroidism, osteoporosis, pancreatitis, submandibular gland lithiasis, nephrolithiasis or osteodystrophy (WO 05/090386).

In order to make a prognostic, a diagnostic, or to evaluate the evolution of a pathological state including the efficiency of a treatment in a subject, there is a need in the art for biomarkers in the field of pain syndroms, hydromineral balance and socio-relational disorders.

SUMMARY

The present invention provides the use of a BPLP gene product, for prognosis, diagnosis or monitoring of a pathological state in a subject.

The present invention is drawn to a method for prognosis, diagnosis or monitoring of a pathological state using a BPLP gene product. The BPLP gene product may be used as a biomarker.

The present invention also provides a method for prognosis, diagnosis or monitoring of a pathological state in a subject comprising the steps of:

a) measuring the quantitative level of a BPLP gene product in a sample obtained from the subject;

b) comparing the quantitative level of the BPLP gene product measured in step a) to a reference value of BPLP gene product;

wherein a significantly higher or lower level of the BPLP gene product compared to the reference value is an indication for the prognosis, diagnosis or evolution of the pathological state.

The BLPP gene products include, but are not limited to, Opiorphin. The BPLP gene products may be used as a biomarker.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 describes the competitive ELISA immuno-assay method used for the quantification of Human Opiorphin.

Figure 2 describes representative Opiorphin-ELISA dose-response curves. (X axis: QRFSR-peptide concentration in log scale; Y axis: percentage of specific binding (B) in the presence of standard peptide / specific binding (B0) in absence of the standard peptide).

Figure 3 describes the specificity of the immuno-assay for Opiorphin (square) compared to Sialorphin (lozenge).

Figure 4 describes representative Opiorphin-ELISA response curves in the saliva from healthy human volunteers (black square: standard QRFSR-peptide (Opiorphin); lozenge: Saliva-Extract n°1a-2 av (basal secretion); circle: Saliva-Extract n°1a-2 ap (stimulated secretion)).

Figure 5 describes the quantification of Opiorphin in male saliva (1st lane, basal secretion; 2nd lane: stimulated secretion; differences between both groups: $P < 0.001$ by Mann-Whitney test) and in female saliva (3rd lane, basal secretion; 4th lane: stimulated secretion. Statistical analysis between groups by Kruskal-Wallis test (KWT)

variance analysis test: P < 0.0001). Difference between both male and female saliva groups under stimulated salivary secretion conditions: P < 0.005 by Mann-Whitney test. Each box represents the whole distribution of values and the inner line indicates the median value.

Figure 6 describes the quantification of Opiorphin in male and female saliva wherein one female saliva sample is excluded from the statistical test compared to the statistical analysis in Figure 5. (1st lane: basal conditions - men; 2nd lane: stimulated conditions – men; 3rd lane: basal conditions – women; 4th lane: stimulated conditions – women). Statistical analysis between groups by Kruskal-Wallis test (KWT) variance analysis test: P < 0.0001.Statistical analysis between two groups by Mann-Whitney test: ** P< 0.01; *** P<0.001.

Figure 7 describes the HPLC chromatographic elution profile and Opiorphin-ELISA analysis of two human plasma extracts: n°1a-11 and 1a-12.

Figure 8 describes the quantification of Opiorphin in the seminal fluid from human volunteers in function of the extraction conditions: in absence or in the presence of 0.1N HCl or 1mM EDTA plus 0.1N HCl. (analysis of variance between groups by KWT: P = 0.0004; and difference between two groups by Mann-Whitney U-test: ** P < 0.01).

Figure 9 describes the quantification of Opiorphin in the seminal fluid from human volunteers. (1st lane: healthy volunteers; 2nd lane: volunteers with congenital bilateral agenesis).

Figure 10 describes the HPLC chromatographic elution profile and Opiorphin-ELISA analysis of two human sperm extracts: n° 3a-13 (healthy) and 3b-02 (agenesis).

Figure 11 describes representative Opiorphin-ELISA response curves in the urine from healthy human volunteers (square: standard QRFSR-peptide (Opiorphin); lozenge: urine extract n°1a-14; circle: urine extract n°1b-15).

Figure 12 describes representative Opiorphin-ELISA response curves in the tears from human healthy volunteers (square: Standard QRFSR-peptide (Opiorphin); lozenge: tears extract n°1a-10; circle: tears extract n° 1b-15).

Figure 13 describes the quantification of Opiorphin in tears from human volunteers (1st lane: women; 2nd lane: men). Difference between both groups by Mann-Whitney U-test ** P<0.01.

DETAILED DESCRIPTION OF THE INVENTION

The endogenous opioid neuropeptides, *i.e.* the enkephalins, play a key role in the negative retro-control of the nociceptive transmission and in the modulation of the activity of cerebral structures governing the attention, the motivation and the adaptive balance of emotional states. The inventors of the present invention have surprisingly discovered that the BPLP gene product Opiorphin protect these endogenous opioid neuropeptides from the inactivation by enkephalinases, and thus on a physiological point of view, Opiorphin may be useful as physiological regulators of enkephalinase activities in Human. More particularly, the BPLP gene products, especially Opiorphin, may be used as new modulators of pathways controlling the painful perception, the emotional balances and the socio-relational behaviors.

Otherwise, the BPLP gene products, especially Opiorphin, as NEP inhibitor may be used as new modulators of pathways controlling hydromineral balances.

Furthermore, the inventors of the present invention have come to the conclusion that on a physiopathological point of view, BPLP gene products, especially Opiorphin,

find a particular application for prognosis, diagnosis or monitoring of pathological states and may be useful as a biological marker with potential prognostic, diagnostic or monitoring value in various pathological situations.

1- BPLP gene products, such as Opiorphin, and uses thereof

It is therefore an aspect of the invention to provide BPLP gene products for use, for instance as a biomarker, in the prognosis, diagnosis or monitoring of a pathological state in a subject.

The human gene BPLP used in accordance with the present invention codes for a polypeptide sequence of 201 amino acids (with the potential signal peptide of secretion) predicted from the cDNA cloned and characterized by Dickinson et al. (Curr Eye Res. 15(4), 377-386, 1996). The gene BPLP is expressed in human lacrimal and submandibular glands. SEQ ID NO.1 shows the cDNA sequence coding for BPLP protein and SEQ ID NO.2 shows the BPLP amino acid sequence. As one skilled in the art may appreciate, the present invention contemplated of using, for instance, the BPLP RNA messengers, the precursor protein and the maturation products of the BPLP precursor protein, i.e. peptides obtained through specific clivage of the BPLP precursor by natural maturases or prohormone converting enzymes, or related mono or paired basic amino acid-cleaving enzymes such as furin, PC convertases or PACE 4 (Seidah et al., Intramolecular Chaperones and Protein Folding, 1995, 9: 181-203). One of its maturation product is Opiorphin, a pentapeptide having the amino acid sequence QRFSR.

The peptides described in the present invention may be prepared in a conventional manner by peptide synthesis in liquid or solid phase by successive couplings of the different amino acid residues to be incorporated (from the N- terminal end to the C-terminal end in liquid phase, or from the C-terminal end to the N-terminal

end in solid phase) wherein the N-terminal ends and the reactive side chains are previously blocked by conventional groups. For solid phase synthesis the technique described by Merrifield may be used in particular. Alternatively, the technique described by Houbenweyl in 1974 may also be used. For more details, reference may be made to WO 98/37100. The peptides according to the present invention may also be obtained using genetic engineering methods.

A patient or a subject is a vertebrate, e.g. a mammal, preferably a human being, regardless of his/her age, sex and general condition. Children and infants are also encompassed except when the studied biological fluid is sperm or milk.

The subject who is evaluated or the test subject may be asymptomatic, may be considered likely to develop the disease or condition or may be symptomatic for the disease or condition. Subjects with a suspicion of a target disorder or subjects who have already shown symptoms of the disease or condition or pathological state can also be tested.

For prognosis or diagnosis, the "reference value" is established by statistical analysis of values obtained from a representative panel of healthy subjects. It may depend from the nature of the sample, the age and/or sex of the subject, the neuroendocrine status etc. It may be predetermined to the measure of the quantitative level of a BPLP gene product, such as Opiorphin, in a subject.

For monitoring, the "reference value" is a value obtained from the subject previously tested.

The control subject is either a healthy subject or, when the evolution of the pathological state of a subject needs to be evaluated, the subject previously tested.

A "biological sample" is a fluid from a subject, including serum, blood, cerebrospinal fluid, urine, milk, tears, saliva or a tissue extract or a tissue or organ biopsy such as brain, spinal cord, bone tissue, kidney, prostate and gonadal glands,

placenta, dental tissue, glandular mucosa of stomach, intestine, salivary gland tissue, mammary glands, for example.

Prognosis or prognostic test refers to the determination or confirmation of a likelihood of a disease or condition to arise in a subject.

Diagnosis or diagnostic test refers to the determination or confirmation of a pathological state, a disease or a condition in a subject.

Monitoring test refers to the determination or confirmation of the evolution of a pathological state, a disease or a condition, including the efficiency of a treatment, in a subject. According to the invention, the pathological state, the disorder, the disease, or the condition refer to any health change relative to a healthy subject.

The pathological states may be, such as, but are not limited to, hyper-algesic syndromes, hypo-algesic syndromes, depressive states and socio-relational behavioral imbalances and hydromineral disorders.

More particularly, the inventors of the present invention provide the use of BPLP gene products, especially Opiorphin, as a biological marker in various biological fluids, such as, but not limited to, saliva for patients developing for instance Mouth Burning Syndrome which is a chronic pain syndrome with unknown etiology; plasma and urines for patients suffering for instance of depression and associated social disorders or neuropathic chronic pain; sperm for patients suffering for instance of erectile dysfunction.

2- Method of use of BPLP gene products, such as Opiorphin .

According to another aspect of the invention, there is provided a method of use of BPLP gene products, such as Opiorphin. More particularly, the method of use provided herein relates to a method for prognosis, diagnosis or monitoring of a pathological state in a subject. The method comprises the steps of:

a) measuring the quantitative level of a BPLP gene product as defined above in a sample obtained from the subject;

b) comparing the quantitative level of the BPLP gene product measured in step a) to a reference value of the BPLP gene product;

wherein a significant higher or lower level of the BPLP gene product compared to the reference value is an indication for the prognosis, diagnosis or evolution of the pathological state.

As used herein, the term "significant" means that the p value is inferior or equal to 0.05. The quantitative level of expression or production of BPLP gene products such as Opiorphin, may be determined by assaying the BPLP RNA messengers, BPLP precursor protein or its maturation products such as the Opiorphin-pentapeptide. Such assay methods comprise contacting a biological sample with a binding partner capable of selectively interacting with a BPLP gene product, such as Opiorphin, present in the sample. The binding partner may be an antibody, which may be polyclonal or monoclonal, or a molecular probe.

Methods for producing antibodies can be easily adapted to produce antibodies useful for the diagnostic, prognostic or monitoring methods according to the invention. For example, the presence or production of the BPLP gene product, such as the Opiorphin peptide, can be detected by contacting a biological sample with an antibody that specifically recognizes the BPLP gene product, such as Opiorphin peptide, e.g. using standard electrophoretic and liquid or solid immunodiagnostic techniques, including immunoassays such as competition, direct reaction, or sandwich type assays. Such assays include, but are not limited to, Western blots; agglutination tests; enzyme-labelled and mediated immunoassays, such as ELISAs; radioimmunoassay such as those using radioiodinated or tritiated BPLP protein or any of its maturation products, such as Opiorphin; immunoelectrophoresis; immunoprecipitation, etc. The reactions generally include revealing labels such as fluorescent, chemiluminescent, radioactive,

enzymatic labels or dye molecules, or other methods for detecting the formation of a complex between the antigen and the antibody or antibodies reacted therewith. Solid supports which can be used in the practice of the invention include supports such as nitrocellulose (e.g., in membrane or microtiter well form); polyvinylchloride (e.g., sheets or microtiter wells); polystyrene latex (e.g., beads or microtiter plates); polyvinylidine fluoride; diazotized paper; nylon membranes; activated beads, magnetically responsive beads, and the like. Thus, in one particular embodiment, the presence of bound BPLP gene products, such as Opiorphin, from a biological sample can be readily detected using a secondary binding agent comprising another antibody, which can be readily conjugated to a detectable enzyme label, such as horseradish peroxidase, alkaline phosphatase or urease, using methods known to those of skill in the art. An appropriate enzyme substrate is then used to generate a detectable signal, such as a chromogenic or fluorogenic signal for example. In other related embodiments, competitive-type ELISA techniques can be practiced using methods known to those skilled in the art. The above-described assay reagents, including the antibodies, can be provided in kits, with suitable instructions and other necessary reagents, in order to conduct immunoassays as described above. The kit can also contain, depending on the particular immunoassay used, suitable labels and other packaged reagents and materials (*i.e.* wash buffers and the like). Standard immunoassays, such as those described above, can be conducted using these kits.

The quantitative level of BPLP gene products may also be measured with the mRNA. An oligonucleotide hybridizing specifically with the mRNA expressed by the BPLP gene or a fragment thereof may be used. The person skilled in the art knows how to prepare such an oligonucleotide, once the BPLP gene sequence or the cDNA sequence is known. For instance, the hybridization may be obtained by using a solution containing: 0.5M sodium phosphate, pH 7,2; 7% sodium dodecylsulfate (SDS); 1mM EDTA; 1% bovine serum albumin; and sonicated salmon sperm DNA: 100mg/mL (US 6, 916,607, Rosinski-Chupin et al.).

Firstly, the level of the BPLP gene product, such as Opiorphin, is measured in a representative panel of healthy subjects, or in the subject for which the evolution of the pathological state is monitored, to obtain the "reference value" of the BPLP gene product, such as Opiorphin.

Secondly, the level of the BPLP gene product, such as Opiorphin, is measured in a subject.

Thirdly, the levels are compared. If they are significantly different ($p \leq 0.05$), either lower or higher, it may be useful for the prognosis, diagnosis or the monitoring of a particular pathological state.

As described in the examples, under the conditions of oral mucosal stimulation by citric acid, salivary secretion of Opiorphin was significantly increased in men: 738±146 compared to basal secretion: 59±17 ng/ml, $p<0.001$, n=6-10; and in women: 247±68 vs basal: 61±23 ng/ml, $p<0.01$, n=10-13.

In a subject with a pathological state, such as, but not limited to, hydromineral imbalance, hyper- or hypo-algesic syndromes, depressive states and socio-relational behavioral imbalances, the level of BPLP gene products, such as Opiorphin, will be different than in a control subject and will be an indication of that pathological state.

The method of use of BPLP gene products, such as Opiorphin, can be used as a marker for evaluation of efficacy of treatments.

In order to determine the evolution of a condition, it may be very useful to test a subject for the expression of a BPLP gene product, such as Opiorphin, and to monitor the effect of a drug or the spreading of the condition, by testing him/her a second time, e.g. a few days, weeks, or months later. In that case the results of the second test are compared with the results of the first test, and in general also with the results obtained with a "healthy" subject. The "control subject" then refers either to the same test subject or to a "healthy subject".

A kit for the prognostic, diagnostic or monitoring test using a BPLP gene product, such as Opiorphin, as a biomarker is also provided. The kit may comprise a binding agent (*e.g.* an antibody or a molecular probe) for specifically recognizing BPLP gene products, such as Opiorphin, and reagents to detect the binding of the agent with BPLP gene products, such as Opiorphin. Control samples (positive and/or negative) can also be included.

The present invention will be more readily understood by referring to the following examples. These examples are illustrative of the wide range of applicability of the present invention and are not intended to limit its scope. Modifications and variations can be made therein without departing from the spirit and scope of the invention. Although any methods and materials similar or equivalent to those described herein can be used in the practice for testing of the present invention, the preferred methods and materials are described.

EXAMPLES

Material and Methods

A. SAMPLING, CONSERVATION AND TRANSPORT OF BIOLOGICAL FLUIDS

The protocol was the following one:

- Biologic samples were taken and collected (*i.e.*, 2-5 ml of saliva, 30 ml of blood, 20 ml of urine by subject) into previously cooled polypropylene tubes (4, 15 or 50 ml) containing a mixture of peptidase inhibitors: Péfabloc 0.4 mM, Aprotinin 1000 KIU/ml, EDTA 1 mM, bestatine 150 µM, leupeptin 1 µM, pepstatin 1 µM (Rougeot and al. Am J Physiol, on 1997). For the sperm the peptidase inhibitors were limited (according to previous validation tests) to Aprotinin and Pefabloc in absence of the anti-coagulant (EDTA).

- They were centrifuged for 15 min at 4000 X g and 4°C and the supernatants were collected (*i.e.*, plasma fraction for the blood);

- All samples were immediately stored at -80 °C; and

- They were transported in dry ice container to the laboratory.

B. DEVELOPMENT OF THE QUANTITATIVE IMMUNO-ASSAY FOR OPIORPHIN

Considering the small size of the Opiorphin (650 Da), an immuno-assay of competitive type was applicable to the quantification of the peptide: *i.e* ., competition between the free antigen to be measured, the Opiorphin QRFSR-peptide (contained in the sample or in the known concentrations of the reference-peptide) and the antigen coated to the support of the 96 well-micro-titration plate (in constant and limited quantity) towards the "anti-QRFSR-peptide" antibodies (in constant and limited quantity) (Fig.1). Y represents the antibody directed against Sialorphin.

On the other hand, for the same reason, the production of the polyclonal "anti-QRFSR" antibodies in the animal (rabbit) required the injection of the antigen, to improve the immunogenicity of the QRFSR-peptide, conjugated in a covalent manner to a carrier molecule (generally a protein from various species excepted human). The immunogen used was ovalbumin-(CO-NH)-YQRFSR (also called OVA-(CO-NH)-YQRFSR).

The specific reaction was revealed by using an universal system supplied by Pierce, *i.e.*, purified immunoglobulin antibodies (IgG-IgM) against rabbit immunoglobulin (IgG) conjugated to the horseradish peroxydase enzyme (HRP) followed by final addition of the chromogenic TMB specific substrate (tetramethylbenzidine).

B.1 FORMAT OF THE COMPETITIVE ELISA IMMUNO-ASSAY FOR OPIORPHIN: - ANTI-YQRFSF ANTIBODY AND - IMMOBILIZATION OF THE Y-(PE)$_{12}$-QRFSR PEPTIDE

The peptide was coated on the plastic support of the micro-titration plate (96-well-ImmunoPlate, Nunc). Different QRFSR-peptide derivatives were tested:

- The Y-QRFSR peptide: the presence of the N-terminal tyrosine residue increases the hydrophobic character of the Opiorphin-peptide, and so as to facilitate its passive adsorption to the plastic support;

- The Y-(PE)$_6$-QRFSR and Y-(PE)$_{12}$-QRFSR peptides: the presence of the 6-polyethylene or 12-polyethylene linker introduced between the tyrosine residue and the QFRSR-peptide sequence facilitates the accessibility of the immobilized peptide to the antibody by rending it more flexible and farther from the coated-support as well as more hydrophobic. As expected, the more the sequence QFRSR is taken away from the support, the more its recognition by the antibody is facilitated. The Opiorphin derivative selected for the plate coating was the Y-(PE)12-QRFSR peptide.

The specific polyclonal anti-QRFSR antibody was generated after administration of the OVA-CO-NH-YQRFSR immunogen to 2 rabbits and a precise follow-up of the specific immune response. The polyclonal antibody selected on the basis of its affinity for Opiorphin was referred as 3RBF-SAB.

The optimized assay conditions were the following ones:

Coating buffer: potassium phosphate buffer at 100 mM and pH 7.1.

Saturating buffer: Tris buffer at 20 mM pH 7.5 + 150 mM NaCl + 0.1% Tween 20 + 0.5% gelatin.

1st Incubation buffer (anti-Opiorphin antibody + sample or reference-peptide): Tris buffer at 200 mM and pH7.5 + 150 mM NaCl + 0.1% Tween 20 + 0.1% bovine serum albumin (BSA).

2nd Incubation buffer (anti-rabbit IgG antibody conjugated to HRP): Tris buffer at 20 mM and pH 7.5 + 150 mM NaCl + 0.1% Tween 20 + 0.1% BSA.

Washing buffer: pure water + 0.1% Tween 20.

The following Table 1 describes in more details the steps of the competitive ELISA immunoassay.

Table 1:

Coating	40 ng of Y-$(PE)_{12}$-QRFSR peptide per 200 µl/wells (96-wells micro-titration plates, Immuno-Plate, Nunc) Incubation over-night (O/N) at +4°C under light agitation
	Washing 5-times
Saturation	250 µl saturation buffer – Incubation 1h. at 22°C
	Washing 5-6 times
1st Immunological Reaction	50 µl of diluted samples or known concentrations of the reference Opiorphin-peptide (from 2 ng/ml to 500 ng/ml) pre-incubated O/N at 4°C in the presence of 50 µl of anti-Opiorphin antibody at 1/80 000
	Incubation on the coated micro-titration plates 1 h. 30 min. at 4°C
	Washing 5-6 times
2nd Immunological Reaction & Revelation	100 µl of the conjugate anti-rabbit IgG-HRP (Pierce) at 1/3 000 Incubation 1 h. at 22°C Washing 5-6 times 100 µl of the HRP chromogenic substrate (Step Ultra TMB-ELISA, Pierce) : Incubation 30 min at 22°C Stop the reaction according to the manufacture conditions (100 µl 4N H_2SO_4) Measure the absorbance at 450 nm wavelength

B.2. Characteristics of the competitive ELISA immuno-assay for Opiorphin

As illustrated in Fig. 2, the competitive-ELISA immuno-assay for Opiorphin, using the anti-YQRFSR antibody Ref. 3RBF-SAB, allowed to perform a reproducible and sensitive assay: the IC50 was 30 ± 9 ng/ml (SD, n=18 standard curves) (3 ng or 5 pmol/assay) and detection limit was 2 ng/ml of Opiorphin QRFSR-peptide (200 pg or 0.3 pmol/assay). Inter-assay variability was less than 10%. In addition, the immuno-assay was highly specific for Opiorphin as the functional related Sialorphin QHNPR-peptide (lozenge) was not recognized by the antibody even at 500 ng/ml final concentration (Fig. 3).

C. EXTRACTION OF OPIORPHIN FROM THE HUMAN BIOLOGICAL SAMPLES

Briefly, after de-freezing at +4°C, the biological samples were treated according to the following conditions:

C.1. Acid-methanol Extraction: 1 volume sample for 4 volumes of 0.1% trifluoroacetic acid (TFA) in methanol at +4°C.

This first step realized the precipitation and the elimination of high molecular weight proteins (*i.e.*, peptidases) which are denatured in acid-methanolic conditions; the soluble low molecular weight molecules (*i.e.*, Opiorphin) in the methanol phase was separated from the precipitate by centrifugation at 4700 rpm during 30 min at +4°C and lyophilized at -110 °C during 48h.

* In the case of sperms and plasmas, samples were previously acidified with HCl at 0.1N final concentration (dissociation of potential ionic interactions between Opiorphin and binding components). In addition, in the case of sperms, samples were previously treated with EDTA at 1mM final concentration (dissociation of Opiorphin from associated cationic metal, *i.e.*, Zn++ concentrated in seminal fluid at about 1.5 mM).

The saliva, tears and milk, dried-extracts were reconstituted in 1 volume of H2O-HPLC (corresponding to the initial volume of samples) centrifuged (4700 rpm, 30 min at

+4°C) and the supernatants were conserved at -80°C until to be analyzed by ELISA for their Opiorphin content.

*In the case of sperms, dried-extracts were reconstituted in 0.2 volume of H2O-HPLC compared to the initial volume of samples (final concentration at 5X).

* In the case of urines, dried-extracts were reconstituted in 0.4 volume of H2O-HPLC compared to the initial volume of samples (final concentration at 2,5X)

C.2. C18-SepPak cartridge Extraction (Waters)

The acidified samples (HCl, 0.1N final concentration) were applied to the pre-activated C18-SepPak cartridges. After washing with H2O-0.1%TFA (5 ml), the components were eluted according to a multi-step gradient of: 5% - 20% - 40% - 60% and 100% methanol-0.1%TFA (5ml each). The successive fractions were collected at 4°C and then lyophilized at -110 °C during 48h.

* This extraction-purification step has been critical in the case of plasmas and milk.

C.3. Reverse phase C18-HPLC Chromatography

The extracts obtained during the procedures described above were applied to the top of the C18/RP-HPLC column (Luna 5µ Phenomenex-USA or ACE 3µ AIT-France (150X4.5 mm)) under TFA 0.1%-H2O solvent conditions.

The various components were eluted and isolated according to their hydrophobic characteristic, in a 30-min linear gradient: from 0% to 80% acetonitrile, and at a 1 ml/min flow rate (Surveyor HPLC system, Thermo-scientific). Each fraction (1 ml) was collected and lyophilized as before and tested for their Opiorphin content by ELISA.

* This HPLC purification step has been critical in the case of plasmas.

Results

OPIORPHIN CONCENTRATION PROFILE IN HUMAN BIOLOGICAL FLUIDS

Example 1: Quantification of Opiorphin in the saliva from healthy human volunteers:

A representative Opiorphin competitive-ELISA assay is shown in Fig. 4; the immuno-reactive curve obtained from successive dilutions of the 1a-2 sample (open circle) was parallel to the dose-response curve corresponding to the reference QRFSR-peptide (black square, concentrations in abscise axis is in log scale). This assesses that the natural Opiorphin-peptide contained in the saliva extract was recognized by the antibody with the same affinity as the synthetic pure peptide.

In saliva samples collected from healthy adult males, Opiorphin was detected in saliva under basal conditions (open lozenge, Fig. 4) as well as but more abundantly under stimulated conditions of salivary secretions (drop of diluted lemon applied to the mouth floor)(open circle).

The statistical analysis of altogether human salivary samples collected from healthy men, revealed that the median concentration of Opiorphin is 50 ng/ml (n=10) in basal conditions, while under conditions of chemical stimulation of salivary secretions, it is evaluated at 870 ng/ml (n=6). So salivary secretion of Opiorphin in men was significantly increased under the conditions of oral mucosal stimulation by citric acid ($p < 0{,}001$, Mann Whitney U-Test, Fig.5).

For the human saliva samples collected from healthy women of similar age, the median concentration of Opiorphin was evaluated at 57 ng/ml in basal conditions (n=11), and at 141 ng/ml in stimulated conditions (n=14). Given the important heterogeneity in Opiorphin salivary levels among women, the difference between the basal and stimulated conditions, was not significant. However, under the chemical

stimulated conditions of salivary secretions, Opiorphin levels in male saliva were significantly higher than those of female saliva ($p < 0,005$ Mann Whitney U-Test, Fig.5).

It is interesting to note that the rate of Sialorphin salivary secretion in male rats under stimulated conditions of the sympathetic nervous system innervating the salivary glands (injection of Noradrenaline-Pilocarpine) was similar (1µg/ml) than that achieved for Opiorphin in men under conditions of chemical stimulation of the mouth nerve endings. On the other hand, as for the secretion rate of salivary Opiorphin in humans, that of salivary Sialorphin in male rats was also significantly higher than that of female rats (Rougeot et al, Eur. Biochem J. .1995).

Strikingly, the important heterogeneity observed in the level of salivary Opiorphin of healthy volunteer women was associated with the fact that one of them had (open circle, Fig. 5) a high salivary concentration of Opiorphin both in basal (1072 ng/ml) and in stimulated conditions (1637 ng/ml). The clinical data associated with this sample revealed that the woman was treated with thyroid hormones. Consequently, this sample was excluded from the final statistical analyses presented in Fig. 6.

Table 2. Results obtained in healthy male and female saliva.

	Mean± Standard Error of Mean (SEM) ng Opiorphin/ml saliva	Number of samples
Healthy men – Basal condition	59±17	10
– Stimulated condition	738±146	6
Healthy women – Basal condition	61±23	10
– Stimulated condition	247 ±68	13

Finally, statistical analyses showed that the salivary secretion of Opiorphin in women is significantly increased under the conditions of oral mucosal stimulation by citric acid ($p < 0,01$, Mann Whitney U-Test, Fig. 6) compared to basal conditions.

Example 2: Quantification of Opiorphin in plasma from healthy human volunteers

A simple acid-methanol extraction procedure (as for saliva) followed by a 5-fold concentration of the dried-extract was insufficient to allow the detection of Opiorphin in plasma, mainly because of the presence of plasmatic components interfering with the ELISA immuno-assay. Thus, the following protocol was applied:

After acidification of the human plasma (HCl, 0.1 N final concentration), the sample was purified on a C18 cartridge-SepPak as described in the chapter "C18-SepPak cartridge Extraction". Recovery of the marker Opiorphin-peptide (QR [3H]FSR)

in fractions eluted with 20% and 40% methanol, was 68% ± 2%, n=4. Fractions were lyophilized, reconstituted in 250 µl H2O-HPLC (40-fold concentration compared to the 10 ml initial plasma volume) and subjected to C18RP-HPLC chromatography according to the procedure described in chapter "Reverse phase C18-HPLC Chromatography". The retention time of the reference Opiorphin-peptide in the chromatographic system (ACE column) was 20 ± 0.2 min, n=4. The HPLC fractions, from 15 to 26 min/ml, corresponding to the samples were collected, lyophilized, reconstituted in 120 µl H2O-HPLC and there Opiorphin content was determined by ELISA. Thus, the final plasmatic samples were concentrated 53-fold compared to the initial sample volume corresponding to 10 ml of human plasma.

As shown in Fig. 7, HPLC elution profile of the human plasma extracts, 1a-11 and 1a-12 demonstrated that Opiorphin is detected at 20 min retention time, which exactly coincide to that of the reference Opiorphin-peptide. This validation step allowed establishing the plasma level of Opiorphin for 4 human plasmas.

Table 3: Results obtained in healthy male and female plasma.

	Mean±SEM ng Opiorphin/ml concentrated extract sample	Number of determinations	Mean±SEM ng Opiorphin /ml initial plasma volume
Healthy men – 1a-11	73±11	9	1.4±0.2
– 1a-12	142±33	9	2.7±0.6
Healthy women – 1b-3	34±3	4	0.6±0.1
– 1b-15	79 ±13	4	1.5±0.2

Interestingly the physiological range of circulating Sialorphin in conscious adult male rats that was established at 1-7 ng/ml (Rougeot and al. Am J Physiol, 1997) is similar to the plasma concentration of Opiorphin in humans established at about 0.6-2.7 ng/ml, for 4 samples.

Example 3: Quantification of Opiorphin in the seminal fluid from human volunteers

As shown on Fig. 8, previous treatment of human sperm samples by EDTA (1mM final concentration) before acid-methanol extraction constituted a crucial step for Opiorphin recognition by the anti-Opiorphin antibody. This suggests that Opiorphin in seminal fluid is associated in a molecular complex involving a cation mineral element, *i.e.*, Zn++ ion contained in the sperm (mM concentration range) and constituting a biochemical prostate marker. Recovery of the marker Opiorphin-peptide added to one sample before acid-methanol extraction was 70%.

Table 4. Results obtained in male seminal fluid.

	Mean±SEM ng Opiorphin/ml sperm	Number of samples	Mean±SEM ng Opiorphin/ejaculation
Healthy volunteers	12.9±1.8	11	43.6±10.2
Volunteers with congenital bilateral agenesis	4.9±1.0	4	6.4±1.6

According to these extraction conditions, the physiological concentration range of Opiorphin in human seminal fluid was established at 12.9±1.8 ng/ml or 43.6±10.2 (SEM, n=11) ng/ejaculate volume (Fig. 9). Interestingly, Opiorphin was also detected in sperm of patients with congenital bilateral agenesis, which is associated with anomalies of the vas deferens and seminal vesicle (inducing infertility although testicular spermatogenesis is intact). Although, their Opiorphin levels (4.9±1.0 ng/ml, n=4) were significantly lower than those of healthy male sperms, the presence of Opiorphin in sperm of these patients suggest that secretion of Opiorphin in the seminal fluid originates for the most part from prostate glands.

In the RP-HPLC chromatographic system using a Luna column, the retention time of the reference Opiorphin-peptide was 22-23 min. Under these chromatographic conditions, the fractionation and ELISA analysis of the sperm extracts, 3a-13 and 3b-02 (Fig. 10) revealed that the immuno-reactive seminal Opiorphin is primarily eluted at 22-23 min retention time, which well-corresponded to the retention time of reference Opiorphin-peptide. Thus, the authentic presence of Opiorphin in human seminal fluid was validated.

Example 4: Quantification of Opiorphin in the urine from human volunteers.

The representative Opiorphin competitive-ELISA assay for the urine samples, 1b-15 and 1a-14 is shown on Fig. 11; the immuno-reactivity obtained from successive dilutions of the urine extracts (black circle and lozenge) was parallel to the dose-response curve corresponding to the reference QRFSR-peptide (black square). This indicates that the natural Opiorphin-peptide contained in these human urines was recognized by the antibody with the same affinity as the synthetic pure peptide.

The percentage of the mean recovery of immunoreactive Opiorphin after incubation of known concentration of synthetic peptide in the presence of urine extract

was 100 ± 19% confirming the absence of interfering urine components in the ELISA test.

Otherwise, the RP-HPLC fractionation and ELISA analysis of the urine extract, 3a-14, showed that immuno-reactive Opiorphin is detected at - 21-22 min retention time coinciding with the retention time of reference Opiorphin-peptide, but also at - 28-29 min retention time as a major immuno-reactive peak. This suggests that Opiorphin in urine fluid is associated to form a more hydrophobic molecular complex. Interestingly, the same kind of HPLC profile was obtained with the sperm extracts in absence of EDTA.

Table 5. Results obtained in healthy male and female urine.

	Mean±SEM ng Opiorphin/ml Urine	Number of determinations
Healthy men volunteers: 1a-14	20±4	4 in duplicate
Healthy women volunteers: 1b-15	14±2	5 in duplicate

The physiological concentration range of Opiorphin in human urine fluid was established at about 14-20 ng/ml, for 2 samples.

Example 5: Quantification of Opiorphin in the tears of human volunteers

Table 6. Results obtained in healthy male and female tears.

	Mean±SEM ng Opiorphin/ml tears	Number of samples
Healthy men volunteers	49±25 (≤ 2 ng/ml)	9 (6)
Healthy women volunteers	317±79 (≤ 2 ng/ml)	14 (1)

The representative Opiorphin competitive-ELISA assay for the tear samples, 1b-15 and 1a-10 is shown in Fig. 12; the immuno-reactive curve obtained from successive dilutions of the 1b-15 tear extract (circle) was parallel to the dose-response curve corresponding to the reference QRFSR-peptide (black square, concentrations in abscise axis is in log scale). This assesses that the natural Opiorphin-peptide contained in the female tears was recognized by the antibody with the same affinity as the synthetic pure peptide. The Opiorphin levels of male tears (*i.e.*, 1a-10, Fig. 12) were for the majority inferior to the detection limit (6 cases out of 9).

The statistical analysis of altogether human tear samples collected from healthy women, showed that the mean concentration of Opiorphin was 317±79 ng/ml (n=14), while that of healthy men was evaluated at 49±25 ng/ml (n=9). Although an important heterogeneity was observed in the level of Opiorphin in tears, it was significantly increased in women compared to men ($p < 0,01$, Mann Whitney U-Test, Fig. 13).

The RP-HPLC fractionation and ELISA analysis of the tear extracts, 1b-04/1b-06/1b-15, showed that immuno-reactive Opiorphin is detected at – 20 min retention time, but also at – 26 retention time and – 29 min retention time as major immuno-reactive peak.

Example 6: Quantification of Opiorphin in the human milk

An important Opiorphin immunoreactivity was found in milk samples collected from seven female volunteers, from two to five week post-delivery. However for the major part of milk extracts (under methanol extraction conditions), the immuno-reactive curve obtained from successive dilutions was not parallel to the dose-response curve of the standard QRFSR-peptide, indicating the presence of milk components interfering with the ELISA immuno-assay. In addition, the RP-HPLC fractionation and ELISA analysis of the milk extract, 2-06, showed that immuno-reactive Opiorphin is detected at – 21 min retention time, but also at – 24-25 retention time as the major immuno-reactive peak and at – 28 min retention time. Thus, the authentic presence of Opiorphin in milk has been assessed after SepPak cartridge extraction of samples in the presence of EDTA, a purification procedure that eliminates the ELISA-interfering milk-components. According to these extraction conditions, the physiological concentration range of Opiorphin in human milk was established at 27±3 ng/ml, n= 9 determinations (in duplicate) for one sample (n°2-06).

Sequence listing

SEQ ID No 1:

```
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ccaaggagca actttaaaga atg aaa tta act ttc ttc ttg ggc ctg ttg gct 113
                       Met Lys Leu Thr Phe Phe Leu Gly Leu Leu Ala
                         1               5                  10
                                                secretory signal peptide
                       <-------------------------------------------
ctt att tca tgt ttc aca ccc agt gag agt caa aga ttc tcc aga aga   161
Leu Ile Ser Cys Phe Thr Pro Ser Glu Ser Gln Arg Phe Ser Arg Arg
            15                  20                  25
                                                    dibasic site
------------------------------------------------>
cca tat cta cct ggc cag ctg cca cca cct cca ctc tac agg cca aga   209
Pro Tyr Leu Pro Gly Gln Leu Pro Pro Pro Pro Leu Tyr Arg Pro Arg
        30                  35                  40

tgg gtt cca cca agt ccc cca cct ccc tat gac tca aga ctt aat tca   257
Trp Val Pro Pro Ser Pro Pro Pro Pro Tyr Asp Ser Arg Leu Asn Ser
    45                  50                  55

cca ctt tct ctt ccc ttt gtc cca ggg cga gtt cca cca tct tct ttc   305
Pro Leu Ser Leu Pro Phe Val Pro Gly Arg Val Pro Pro Ser Ser Phe
60                  65                  70                  75

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Ser Arg Phe Ser Gln Ala Val Ile Leu Ser Gln Leu Phe Pro Leu Glu
                80                  85                  90

tct att aga caa cct cga ctc ttt ccg ggt tat cca aac cta cat ttc   401
Ser Ile Arg Gln Pro Arg Leu Phe Pro Gly Tyr Pro Asn Leu His Phe
            95                  100                 105

cca cta aga cct tac tat gta gga cct att agg ata tta aaa ccc cca   449
Pro Leu Arg Pro Tyr Tyr Val Gly Pro Ile Arg Ile Leu Lys Pro Pro
        110                 115                 120

ttt cct cct att cct ttt ttt ctt gct att tac ctt cct atc tct aac   497
Phe Pro Pro Ile Pro Phe Phe Leu Ala Ile Tyr Leu Pro Ile Ser Asn
```

```
      125                 130                 135

cct gag ccc caa ata aac atc acc acc gca gat aca aca atc acc aca   545
Pro Glu Pro Gln Ile Asn Ile Thr Thr Ala Asp Thr Thr Ile Thr Thr
140                 145                 150                 155

aat ccc ccc acc act gca aca gca acc acc agg cac ttc cac aaa acc   593
Asn Pro Pro Thr Thr Ala Thr Ala Thr Thr Arg His Phe His Lys Thr
                160                 165                 170

cac aat gac gat cag ctc ctc aac agt acc tat ctc ttc aac acc aga   641
His Asn Asp Asp Gln Leu Leu Asn Ser Thr Tyr Leu Phe Asn Thr Arg
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gcc tgc cac ctc cat atc agc agc aac ccc cgc agc atc tac tga       686
Ala Cys His Leu His Ile Ser Ser Asn Pro Arg Ser Ile Tyr
        190                 195                 200

aaatactact caaattctcg ccaaccgtcc tcacacagta ttgctcaatg ccactgtcca 746

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actctttgcc atttttggtt gaacatgcaa taaatgatat tttccaaact gctctgatat 866

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SEQ ID No 2:

Met Lys Leu Thr Phe Phe Leu Gly Leu Leu Ala Leu Ile Ser Cys Phe
1 5 10 15

Thr Pro Ser Glu Ser Gln Arg Phe Ser Arg Arg Pro Tyr Leu Pro Gly
20 25 30

Maturation product Opiorphin

Gln Leu Pro Pro Pro Pro Leu Tyr Arg Pro Arg Trp Val Pro Pro Ser
35 40 45

Pro Pro Pro Pro Tyr Asp Ser Arg Leu Asn Ser Pro Leu Ser Leu Pro
50 55 60

Phe Val Pro Gly Arg Val Pro Pro Ser Ser Phe Ser Arg Phe Ser Gln
65 70 75 80

Ala Val Ile Leu Ser Gln Leu Phe Pro Leu Glu Ser Ile Arg Gln Pro
85 90 95

Arg Leu Phe Pro Gly Tyr Pro Asn Leu His Phe Pro Leu Arg Pro Tyr
100 105 110

Tyr Val Gly Pro Ile Arg Ile Leu Lys Pro Pro Phe Pro Pro Ile Pro
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Phe Phe Leu Ala Ile Tyr Leu Pro Ile Ser Asn Pro Glu Pro Gln Ile
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Ala Thr Ala Thr Thr Arg His Phe His Lys Thr His Asn Asp Asp Gln
165 170 175

Leu Leu Asn Ser Thr Tyr Leu Phe Asn Thr Arg Ala Cys His Leu His
180 185 190

Ile Ser Ser Asn Pro Arg Ser Ile Tyr
195 200

CLAIMS:

1. Use of a Basic Prolin-rich Lacrimal Protein gene product for prognosis, diagnosis or monitoring of a pathological state in a subject.

2. The use according to claim 1, wherein the BPLP gene product is the QRFSR-peptide Opiorphin.

3. The use according to any one of claims 1 to 2, wherein the BPLP gene product is used as a biomarker.

4. A method for prognosis, diagnosis or monitoring of a pathological state in a subject comprising the steps of :

a) measuring the quantitative level of a BPLP gene product, in a sample obtained from the subject;

b) comparing the quantitative level of the BPLP gene product measured in step a) to a reference value of the BPLP gene product;

wherein a significantly higher or lower level of the BPLP gene product compared to the reference value is an indication for the prognosis, diagnosis or evolution of the pathological state.

5. The method according to claim 4, wherein the sample is obtained from a biological fluid or a tissue of the subject.

6. The method according to claim 5, wherein the biological fluid is blood, plasma, saliva, urines, tears, sperm or milk.

7. The method according to claim 4, wherein the BPLP gene product is a BPLP-protein maturation product, especially the QRFSR-peptide Opiorphin.

8. The method according to claim 6, wherein the BPLP-protein maturation product, especially Opiorphin, is extracted by acid-methanol extraction, C18-SepPak cartridge extraction or reverse phase C18-HPLC chromatography.

9. The method according to claim 8, wherein the sample is saliva, sperm, tears, or urines and the BPLP-protein maturation product, especially Opiorphin, is extracted by acid-methanol extraction.

10. The method according to claim 8, wherein the sample is plasma or milk and the BPLP-protein maturation product, especially Opiorphin, is extracted by C18-SepPak cartridge extraction.

11. The method according to claim 8, wherein the sample is plasma and the BPLP-protein maturation product, especially Opiorphin, is extracted by reverse phase C18-HPLC chromatography.

12. The method according to any one of claims 5 to 11, wherein the quantitative level of the BPLP gene product is measured with an immuno-assay.

13. The method of claim 12, wherein the immuno-assay is a competitive ELISA.

14. The method according to claim 13, wherein the immuno-assay comprises a polyclonal anti-QRFSR antibody.

15. The method according to claim 14, wherein the polyclonal anti-QRFSR antibody is 3RBF-SAB.

16. The method according to claim 5, wherein the sample is a tissue and the BPLP gene product is mRNA.

17. The use according to claim 3, wherein the level of the BPLP gene product is measurable in a biological fluid or a tissue of the subject.

18. The use according to claim 17, wherein the biological fluid is blood, plasma, saliva, urines, tears, sperm or milk.

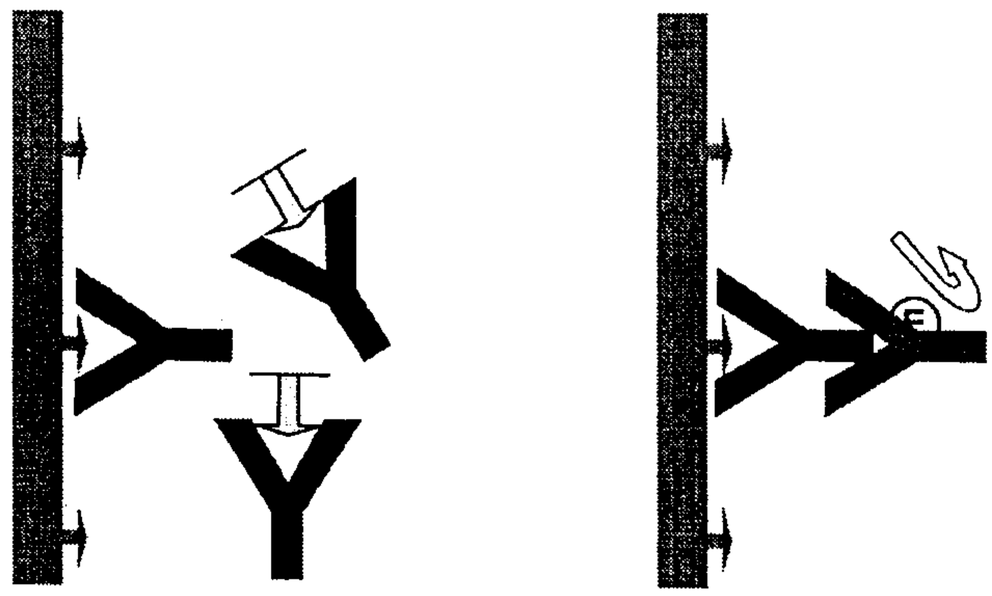
19. A kit for a prognostic, diagnostic or monitoring test, wherein said kit comprises:

- a binding agent for specifically recognizing a BPLP gene product, such as Opiorphin,

- reagents to detect the binding of the agent with the BPLP gene product, such as Opiorphin, and

- optionally, positive and/or negative control sample(s).

20. The kit according to claim 19 wherein the binding agent is an antibody or a molecular probe.



Y Anti-Opiorphin antibodies

Anti-IgG antibodies conjuguated to HRP

Immobilized Opiorphin

Opiorphin

HRP substrate

Figure 1

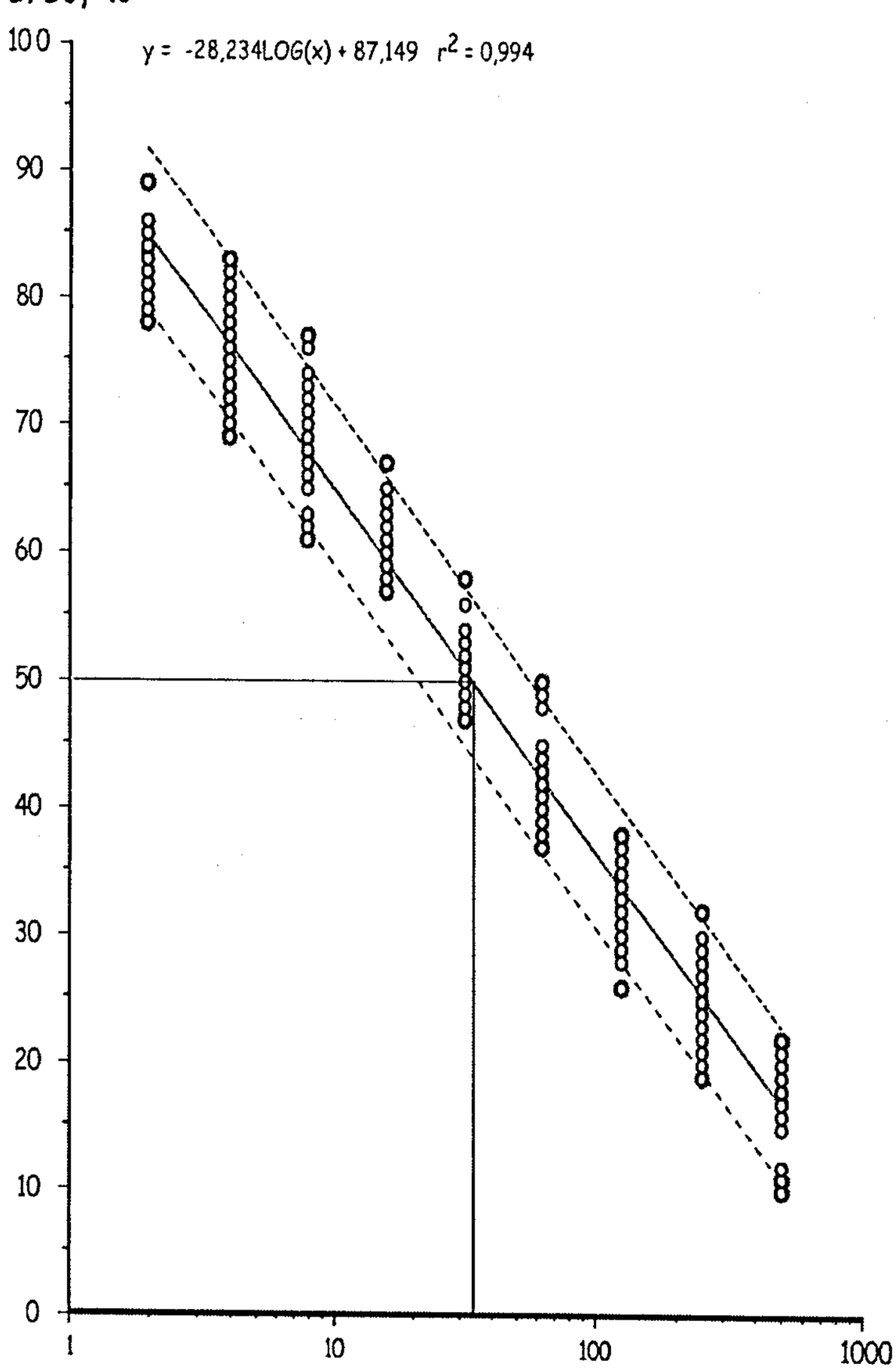
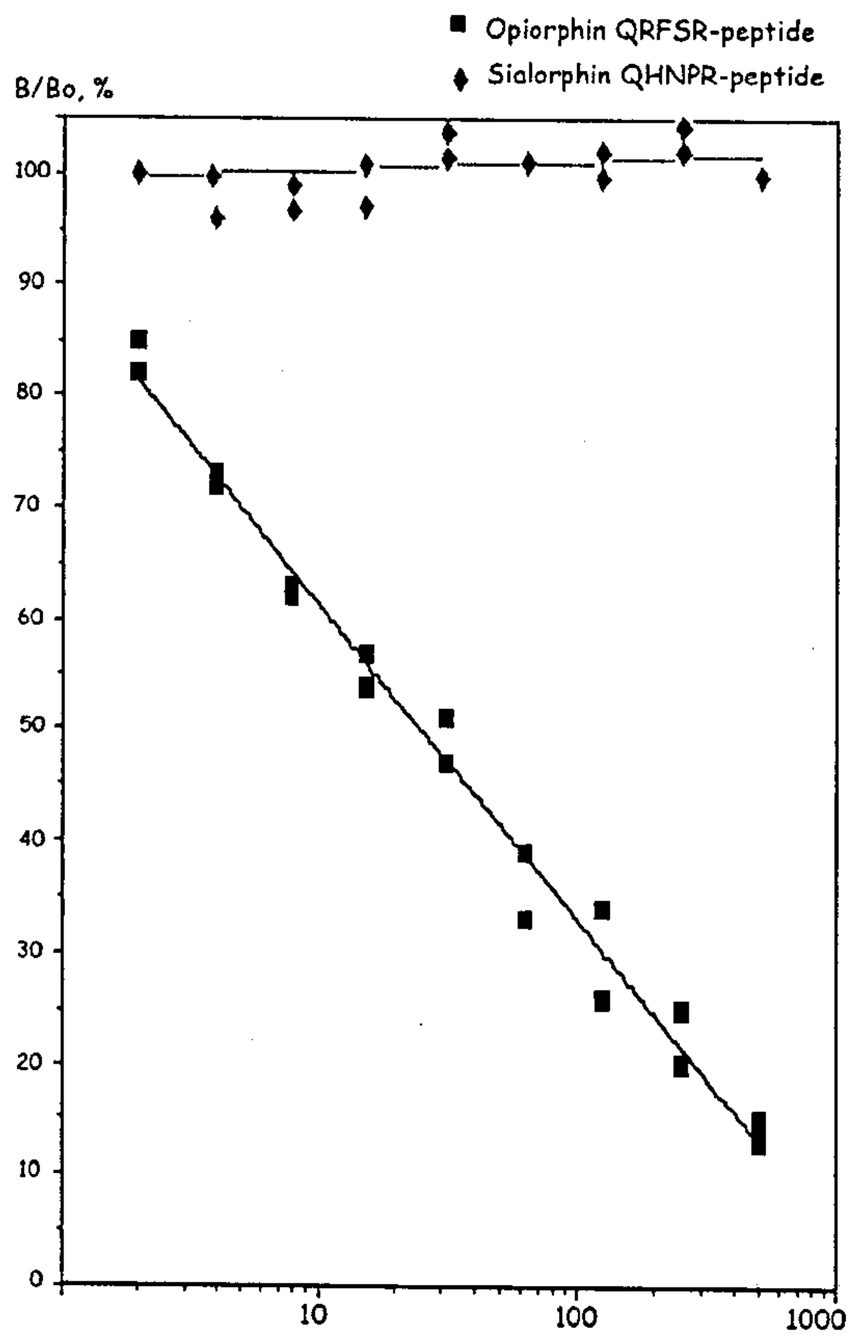

B/Bo, %
y = -28,234LOG(x) + 87,149 r2 = 0,994
100
90
80
70
60
50
40
30
20
10
0
1
10
100
1000
[QRFSR], ng/ml (log scale)

Figure 2


Opiorphin QRFSR-peptide
Sialorphin QHNPR-peptide
B/Bo, %
100
90
80
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60
50
40
30
20
10
0
10
100
1000
[Peptide], ng/ml (log scale)

Figure 3

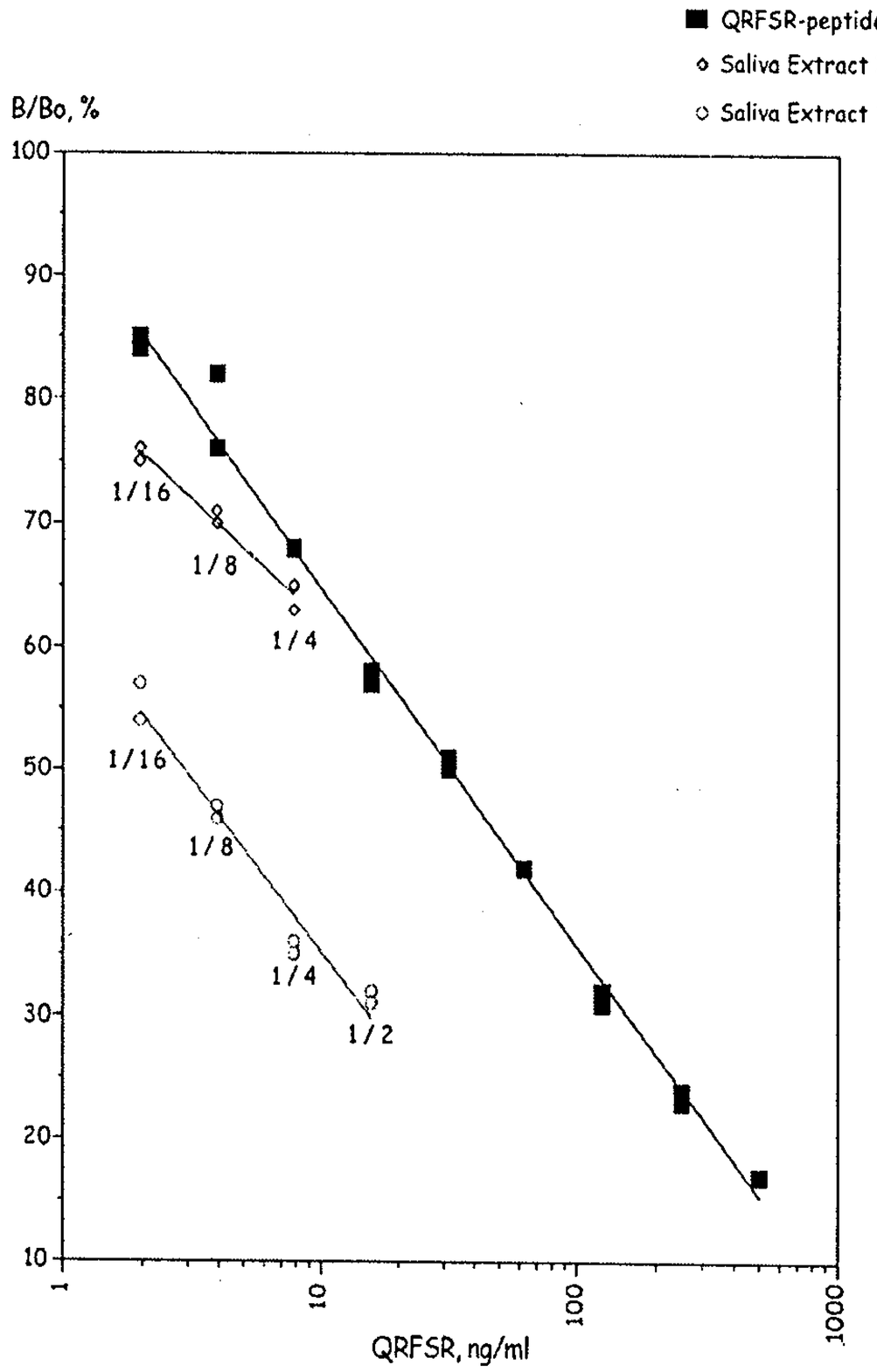
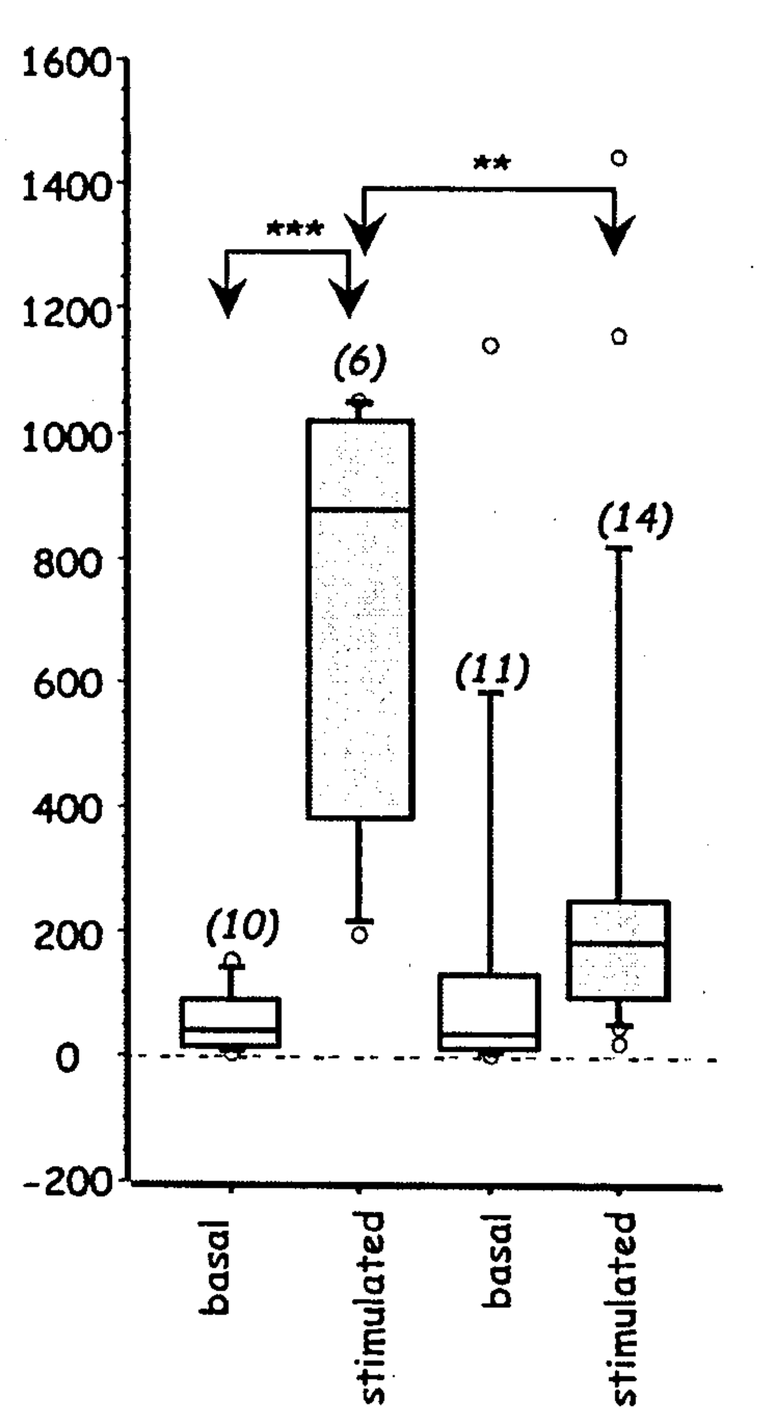

QRFSR-peptide
Saliva Extract 1a-2 av
Saliva Extract 1a-2 ap
B/Bo, %
100
90
80
70
60
50
40
30
20
10
1/16
1/8
1/4
1/16
1/8
1/4
1/2
1
10
100
1000
QRFSR, ng/ml

Figure 4



KWT variance analysis Test: P<0.0001
*Mann-Whitney test: ** P<0.005*
**** P<0.001*

Figure 5

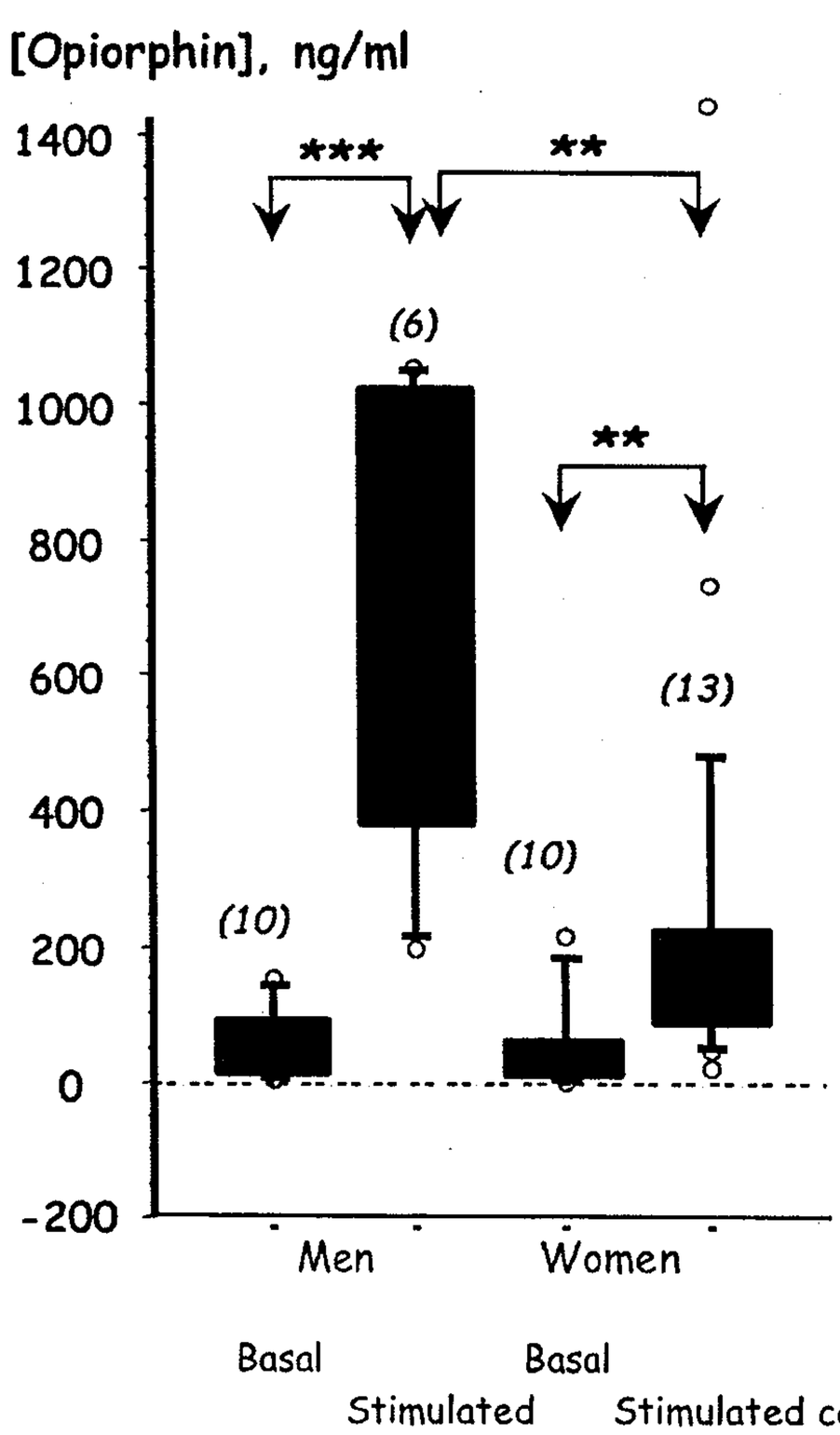


Figure 6

KWT: P<0.0001
*Mann-Whitney test:** P<0.01*** P<0.001*

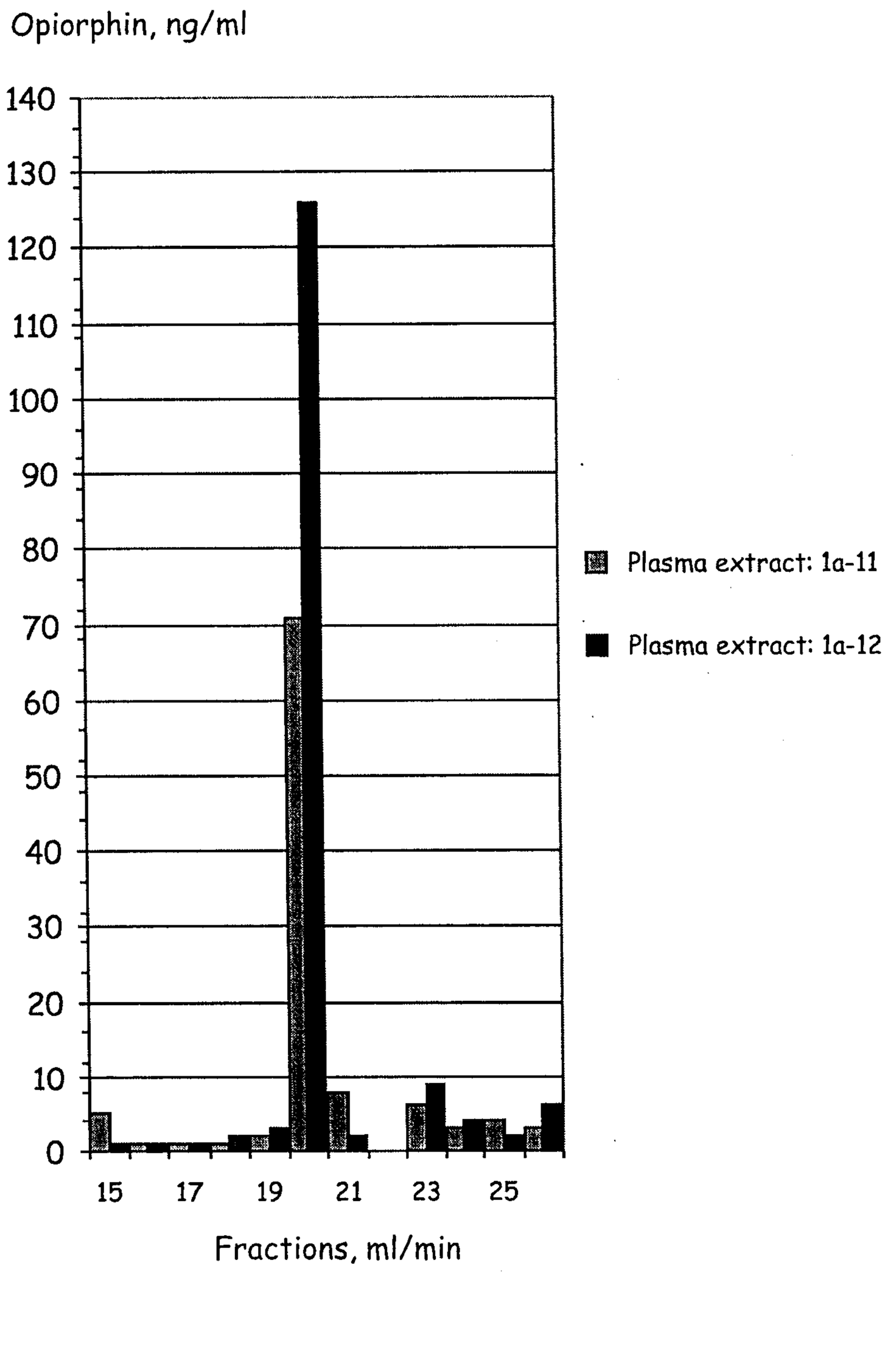

Opiorphin, ng/ml
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
15
17
19
21
23
25
Fractions, ml/min
Plasma extract: 1a-11
Plasma extract: 1a-12

Figure 7

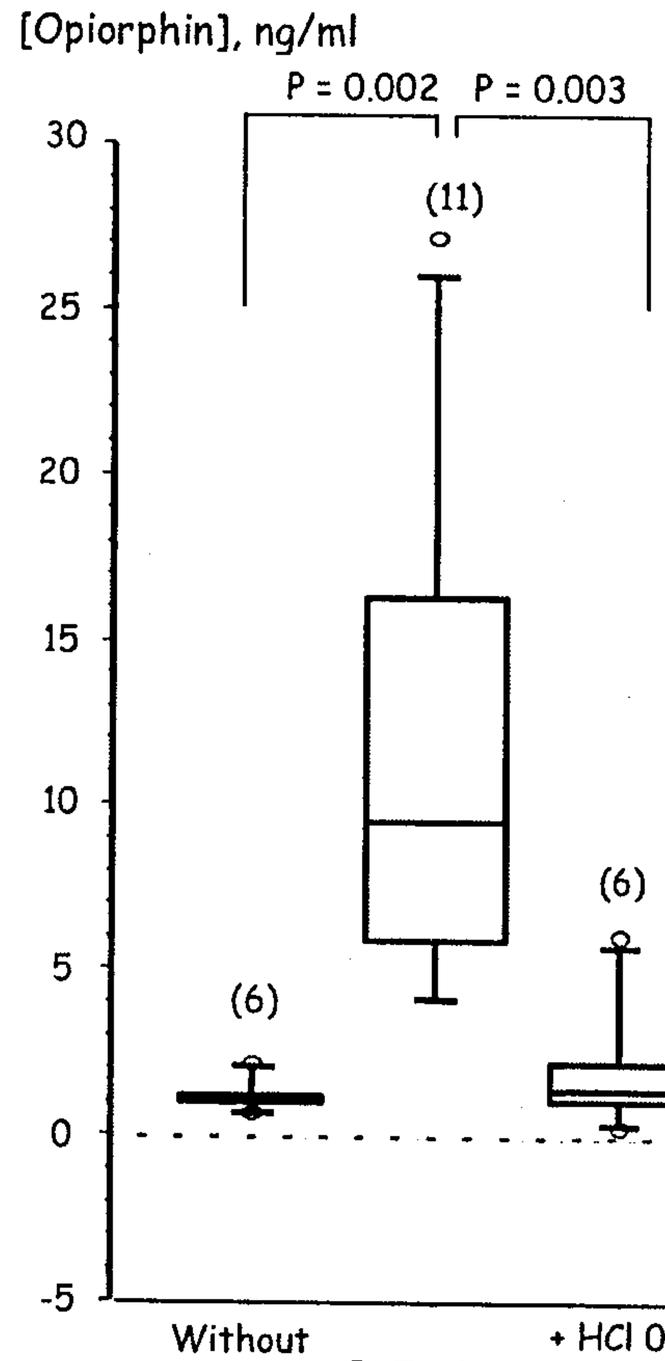

[Opiorphin], ng/ml
P = 0.002
P = 0.003
30
25
20
15
10
5
0
-5
(11)
(6)
(6)
KWT: P=0.0004
Mann-Whitney U-test:
** P<0.01
Without
+ HCl 0.1N
+ EDTA 1 mM
+ HCl 0.1N
Treatment before MeOH-TFA extraction

Figure 8

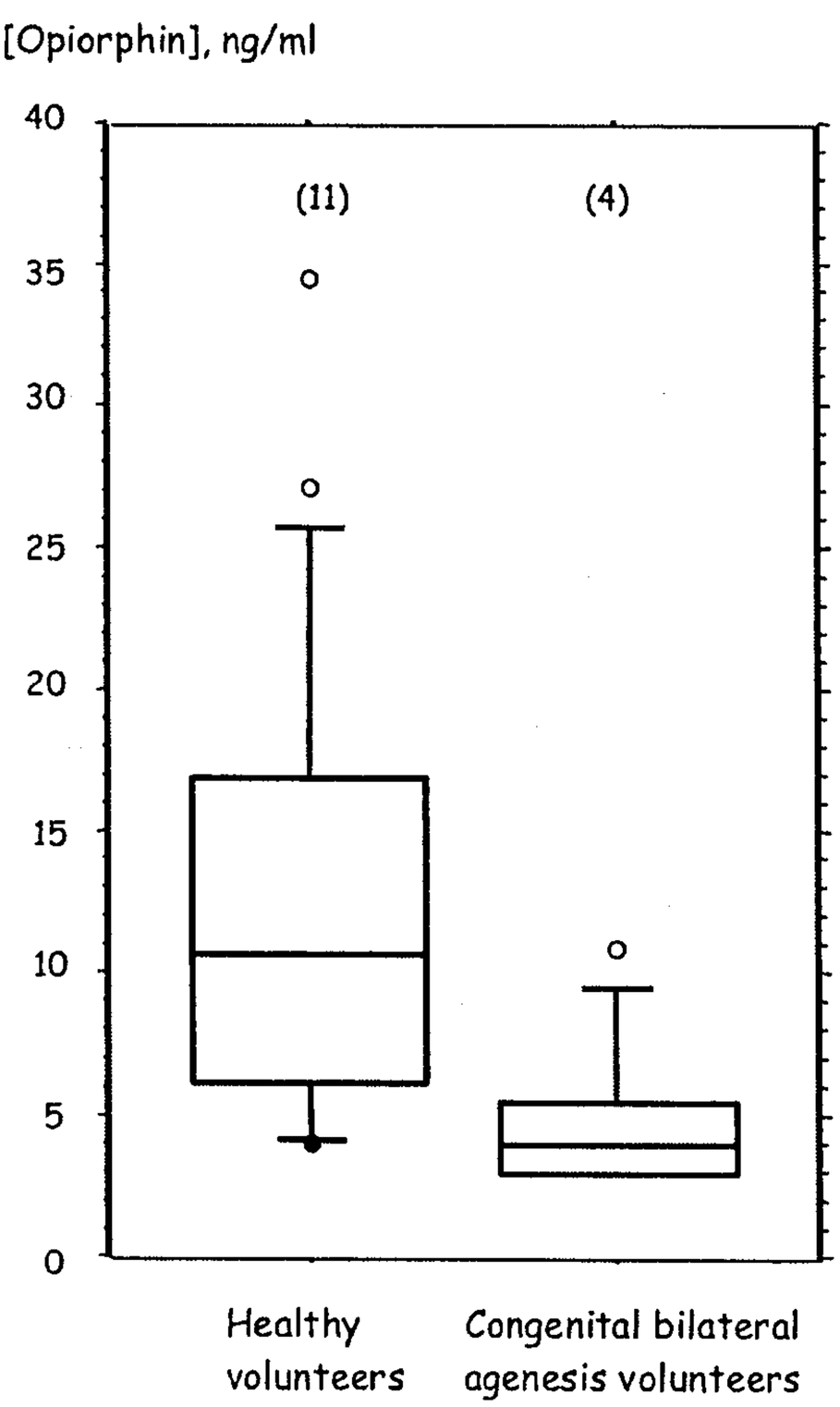

[Opiorphin], ng/ml
40
35
30
25
20
15
10
5
0
(11)
(4)
Healthy volunteers
Congenital bilateral agenesis volunteers

Figure 9

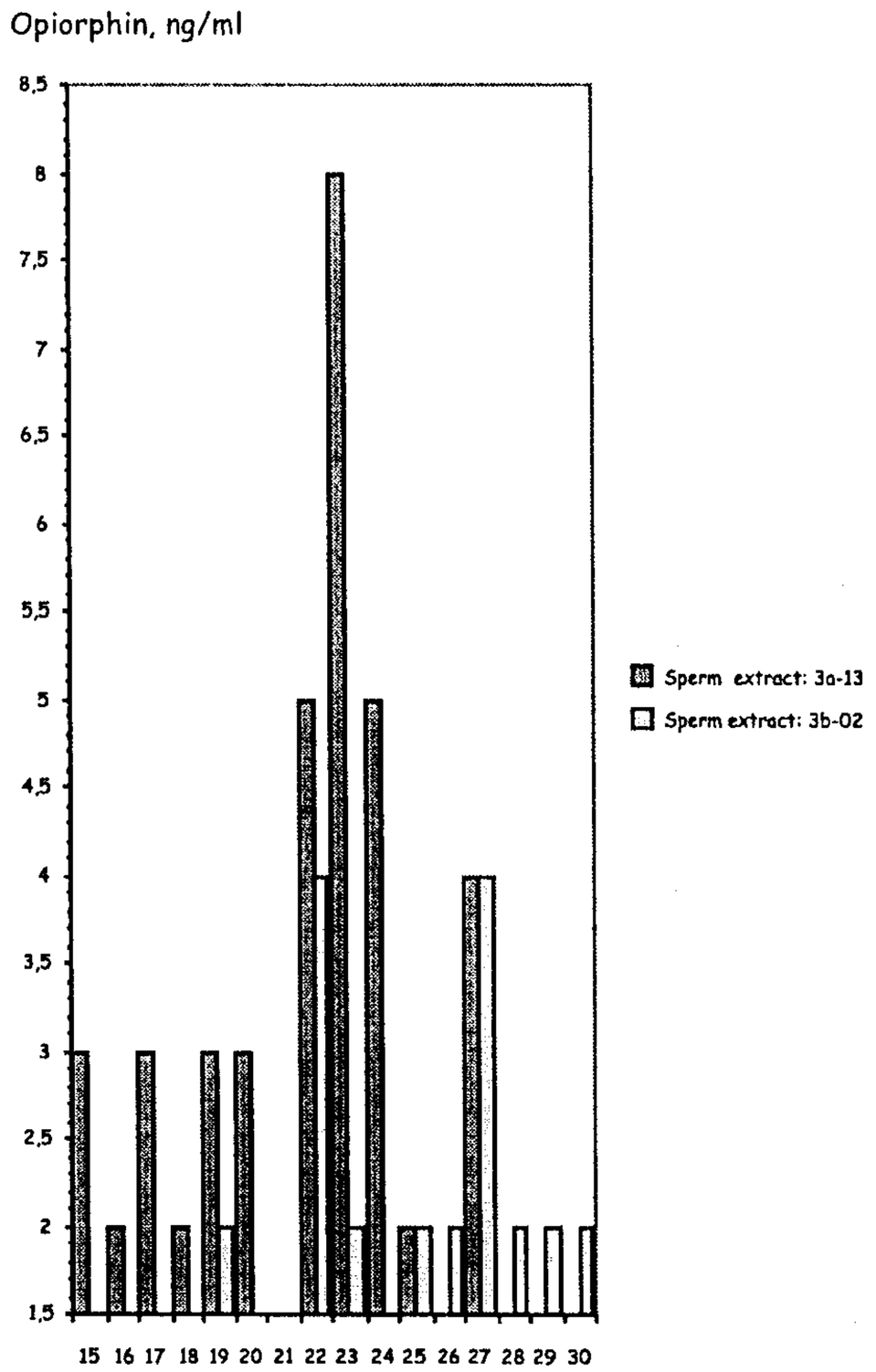

Opiorphin, ng/ml
8,5
8
7,5
7
6,5
6
5,5
5
4,5
4
3,5
3
2,5
2
1,5
Sperm extract: 3a-13
Sperm extract: 3b-02
15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
Fractions, min/ml

Figure 10

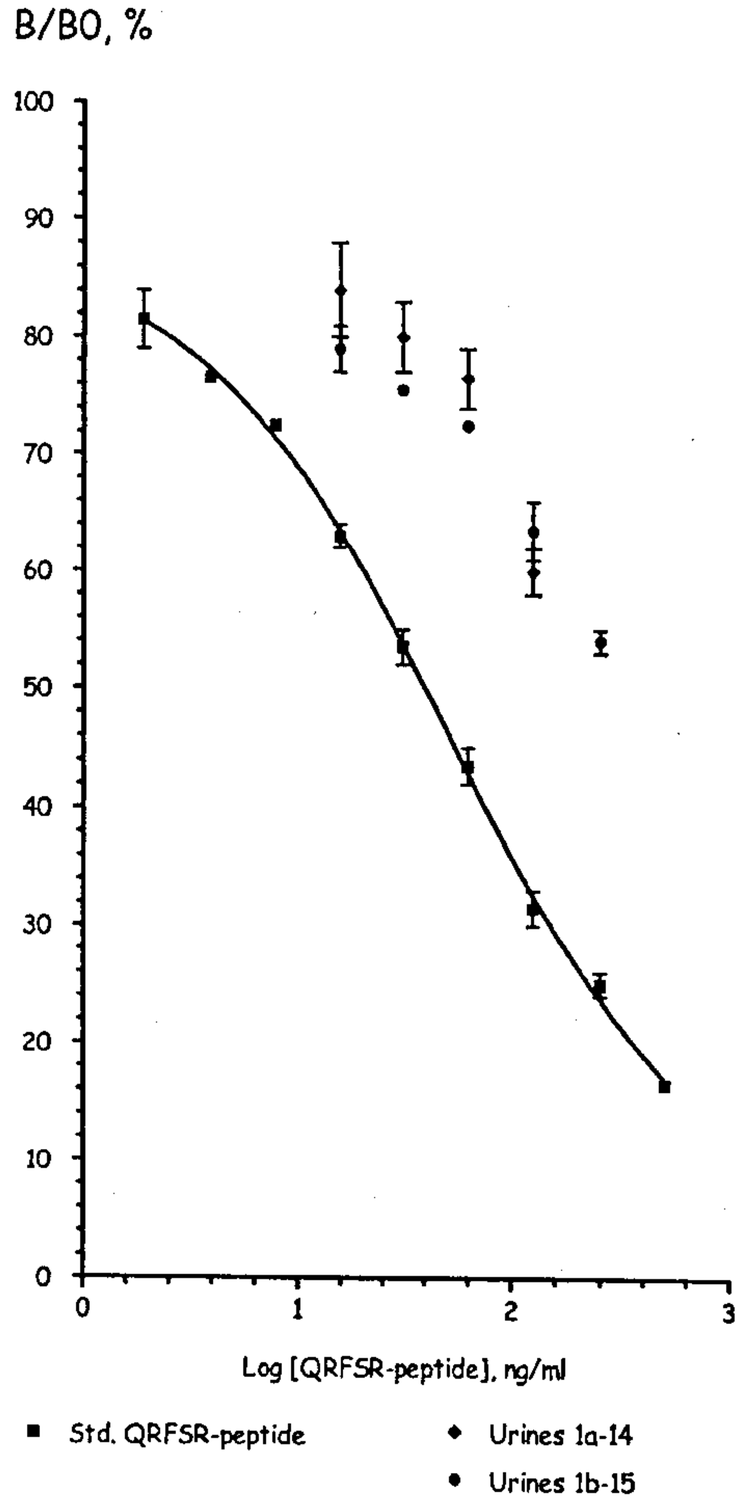

B/BO, %
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
Log [QRFSR-peptide], ng/ml
Std. QRFSR-peptide
Urines 1a-14
Urines 1b-15

Figure 11

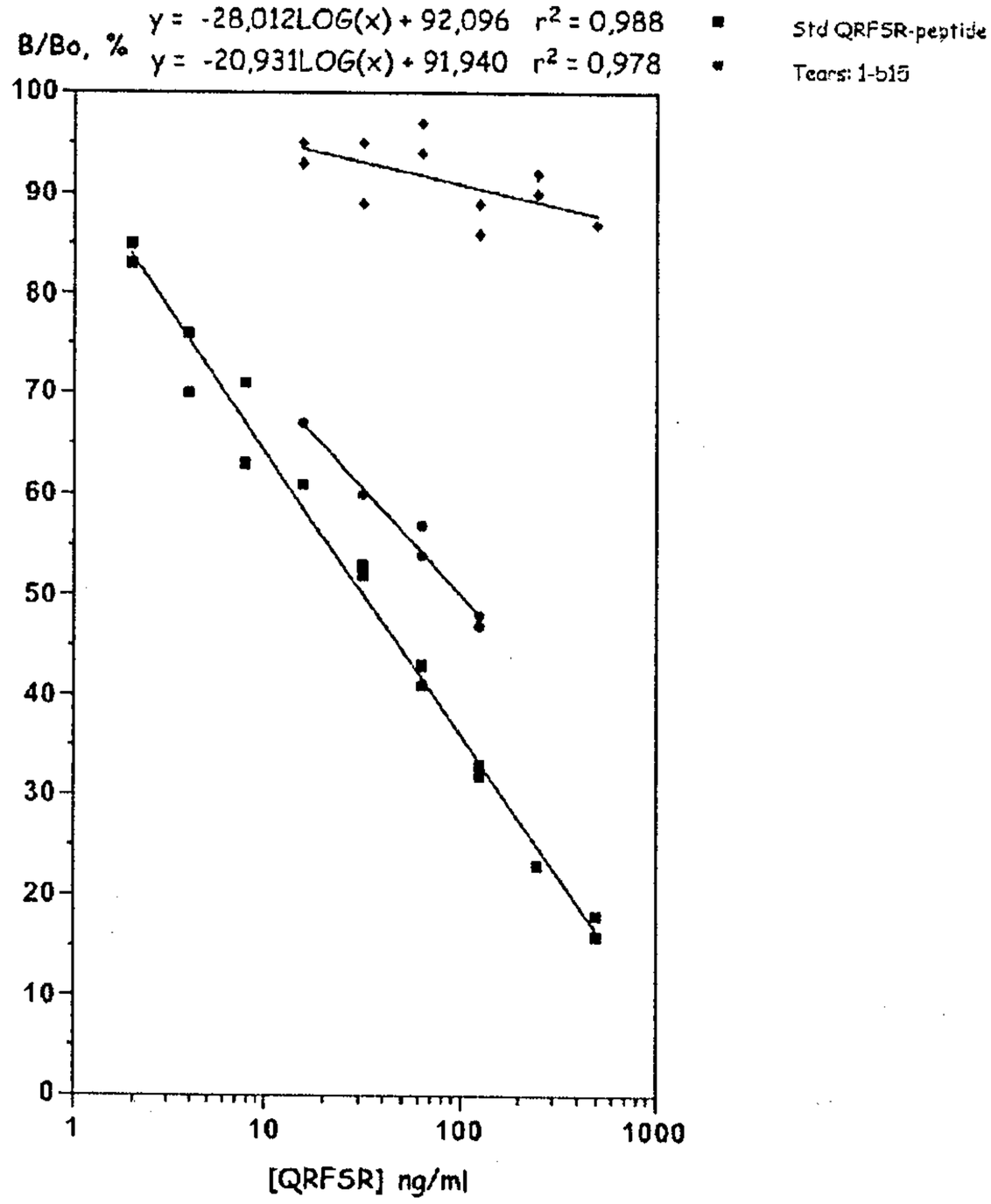
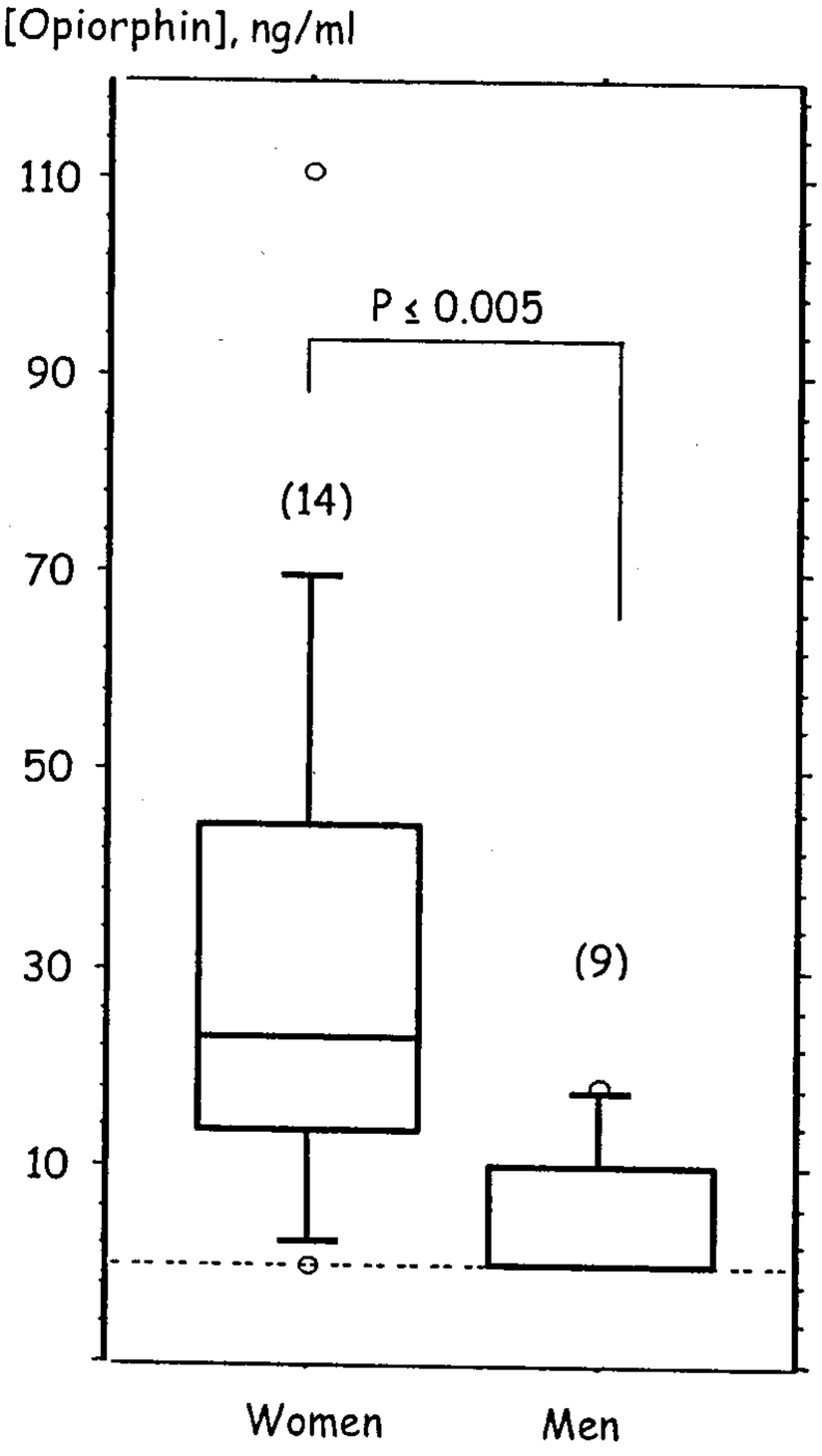
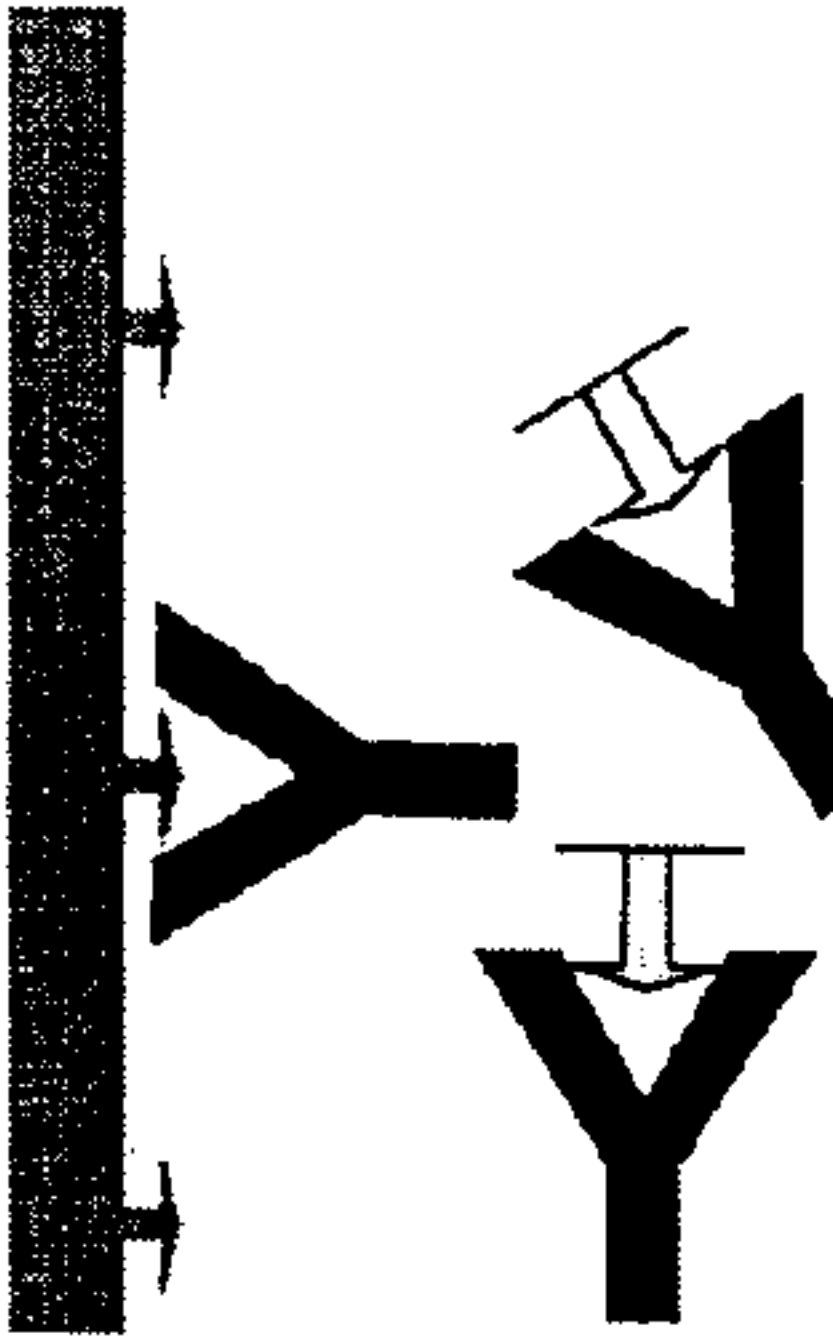

y = -28,012LOG(x) + 92,096 r² = 0,988
y = -20,931LOG(x) + 91,940 r² = 0,978
B/Bo, %
Std QRFSR-peptide
Tears: 1-b15
100
90
80
70
60
50
40
30
20
10
0
1
10
100
1000
[QRFSR] ng/ml

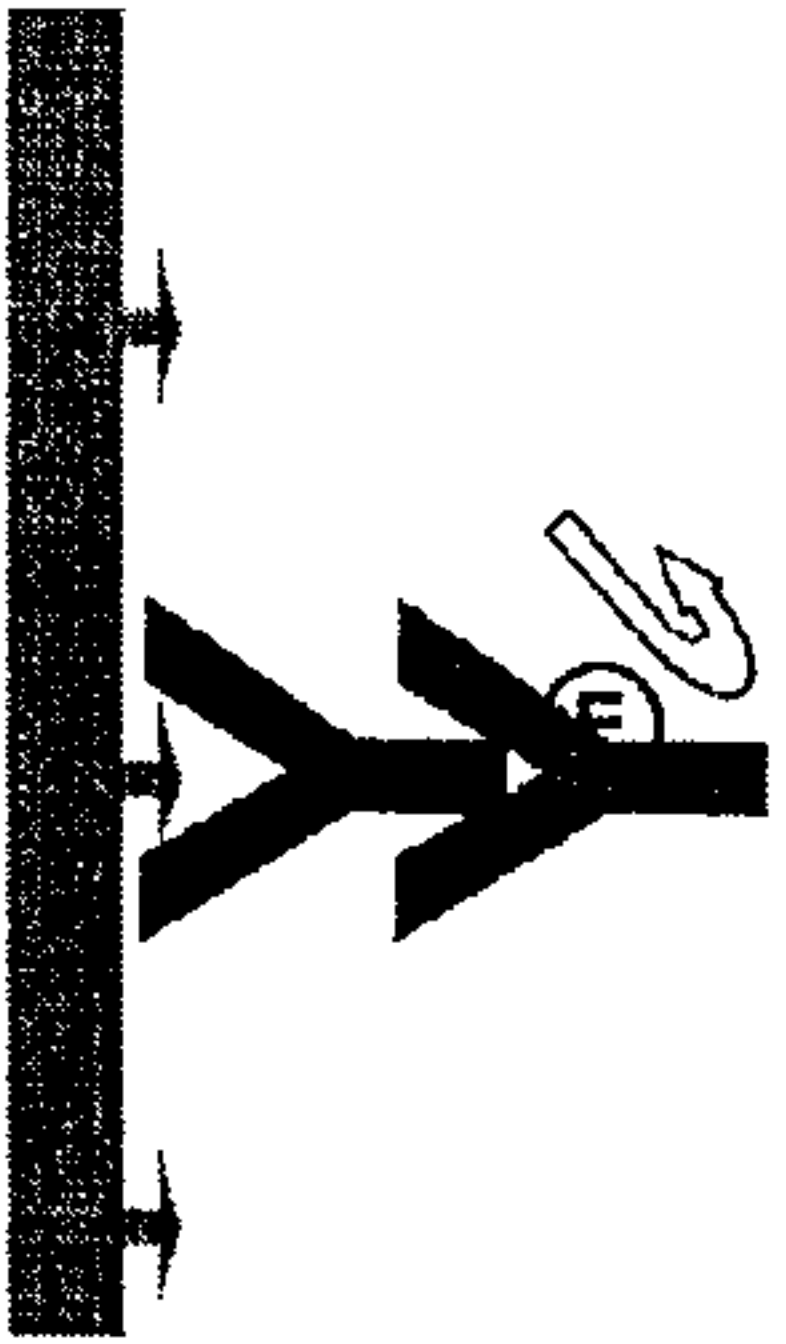
Figure 12



Mann-Whitney U-test
*** P<0.01*

Figure 13





Y Anti-Opiorphin antibodies

Anti-IgG antibodies conjuguated to HRP

Immobilized Opiorphin

Opiorphin

HRP substrate