



(12) **DEMANDE DE BREVET CANADIEN
CANADIAN PATENT APPLICATION**
(13) **A1**

(22) Date de dépôt/Filing Date: 2010/05/14
(41) Mise à la disp. pub./Open to Public Insp.: 2010/11/18
(62) Demande originale/Original Application: 2 761 628
(30) Priorité/Priority: 2009/05/14 (IT PD2009A 000134)

(51) Cl.Int./Int.Cl. *A61K 31/737* (2006.01),
A61K 31/196 (2006.01), *A61K 31/728* (2006.01),
A61K 47/36 (2006.01), *A61P 17/00* (2006.01)
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(54) Titre : NOUVEAUX MEDICAMENTS A USAGE TOPIQUE A BASE D'ACIDE HYALURONIQUE SULFATE COMME
AGENT D'ACTIVATION OU D'INHIBITION DE L'ACTIVITE CYTOKINIQUE
(54) Title: NEW MEDICINES FOR TOPIC USE BASED ON SULFATED HYALURONIC ACID AS ACTIVATING OR
INHIBITING AGENT OF THE CYTOKINE ACTIVITY

(57) **Abrégé/Abstract:**

The object of the present invention relates to the new and surprising use of sulfated hyaluronic acid (HAS) as regulator agent of the cytokine activity (pro- and anti- inflammatory) and consequently the use of HAS for the preparation of a new medicine for topic use in the prevention and treatment of pathologies associated with the activation and/or deficiency of cytokines of a pro- and anti-inflammatory nature. The Applicant has in fact discovered the exclusive capacity of HAS in modulating the activity of these particular proteins, it has studied the action mechanism and demonstrated the substantial difference between the different sulfated types known in the state of the art, but above all it has demonstrated an unexpectedly high activity of HAS vs different types and strains of Herpes virus, Cytomegalovirus and the virus of vesicular stomatitis. Finally, a further object of the present invention is the use of HAS as a skin absorption promoter of drugs of an anti-inflammatory nature.

ABSTRACT

The object of the present invention relates to the new and surprising use of sulfated hyaluronic acid (HAS) as regulator agent of the cytokine activity (pro- and anti-inflammatory) and consequently the use of HAS for the preparation of a new medicine for topic use in the prevention and treatment of pathologies associated with the activation and/or deficiency of cytokines of a pro- and anti-inflammatory nature. The Applicant has in fact discovered the exclusive capacity of HAS in modulating the activity of these particular proteins, it has studied the action mechanism and demonstrated the substantial difference between the different sulfated types known in the state of the art, but above all it has demonstrated an unexpectedly high activity of HAS vs different types and strains of Herpes virus, Cytomegalovirus and the virus of vesicular stomatitis. Finally, a further object of the present invention is the use of HAS as a skin absorption promoter of drugs of an anti-inflammatory nature.

NEW MEDICINES FOR TOPIC USE BASED ON SULFATED HYALURONIC
ACID AS ACTIVATING OR INHIBITING AGENT OF THE CYTOKINE
ACTIVITY

This application has been divided out of Canadian Patent
5 Application Serial No. 2,761,628 filed internationally on
May 14, 2010 as International Application Serial No.
PCT/EP2010/003050 and published internationally on
November 18, 2010 as WO 2010/130468.

Field of the invention

10 For many years now, scientific/patent literature has been
studying and describing sulfated hyaluronic acid which is
obtained starting from hyaluronic acid (HA) suitably sul-
fated according to what is described in the state of the
art (EP0940410B1 and EP0702699B1), to which anticoagulant
15 effects are attributed. HAS can also be obtained by the
de-acetylation and subsequent sulfation of glucosamine of
HA (defined as HA-NS) (EP0971961B1), for the production
of surgical articles and pharmaceutical compositions. Pa-
tents EP0754460B1 and EP1385492B1 are also known, in
20 which the use of HAS is described in pathologies such as,
for example, ARDS (serious respiratory insufficiency),
articular rheumatism and rheumatoid arthritis. An object
of the present invention relates to the new and surpris-
ing cutaneous use of HAS as regulator agent of the cyto-
25 kine activity, as the Applicant has discovered the exclu-

sive capacity of HAS of modulating the activity of particular cytokines (both pro- and anti-inflammatory), it has studied its action mechanism and revealed the substantial difference between the two types of sulfated
5 product (HAS and HA-NS), but above all the Applicant has surprisingly found an unexpectedly high activity vs different types and strains of Herpes virus, Cytomegalovirus and the virus of vesicular stomatitis.

Finally, the present invention further relates to
10 the use of HAS as a skin absorption promoter of drugs of an anti-inflammatory and hormonal nature.

Since 1970, scientists have understood that selected populations of lymphoid cells can produce and release into the circulatory bed, molecules of a protein nature not
15 assimilable to antibodies, defined with the term "cytokines". They represent a new type of "hormone", capable of acting on different cell targets in numerous regions of the body.

The progression of scientific knowledge relating to
20 the synthesis and biological/biochemical functions of these proteins, has altered the "old" vision of the immune system (I.S.) of the same scientific world and has opened up new horizons in understanding its numerous functions, thus creating new prospects for the treatment
25 of different pathologies, topic and/or systemic, also

comprising new therapeutic possibilities relating to the immunotherapy of cancer.

The central cell of the I.S., is the lymphocyte, it represents about 20% of all the white corpuscles and, on 5 the basis of its various functions, forms 3 groups: lym-

phocyte B, lymphocyte T and killer lymphocyte. Many cytokines are soluble proteins produced by lymphocytes and/or monocytes, capable of acting against other cells/tissues also situated very far from their production site. They
5 have immunological functions, in fact, and also regulation functions in the synthesis of other cytokines on the part of different cells of the I.S. or target cells involved in the cascade of reactions initiated by the I.S..

Numerous different cytokines have been studied so
10 far, also having numerous different acronyms, but those studied in particular by the Applicant are: Interleukin 1 and 2, Interleukin 6, 7 and 12, hereafter defined as IL-1, IL-2, IL-6, IL-7 and IL-12 which, with TNF, are defined as cytokines of an inflammatory nature, whereas In-
15 terleukin 10 (IL-10) on the contrary, is a cytokine with strong anti-inflammatory properties.

The first cytokine to be studied was definitely IL-1: present in two forms α and β , it is a powerful inducer of pro-inflammatory processes (systemic and/or cutaneous). It is mainly produced by lymphocytes B, T and
20 macrophages after bacterial stimulus or stimulation on the part of other agents including other cytokines; it is also secreted from peripheral neutrophils, endothelial, epithelial and smooth muscle cells, fibroblasts, Langerhans cells of the skin, osteoclasts, synoviocytes and
25

many other types of cell. Both forms bind the same receptor and have very similar, if not identical, biological activities. Many of their pro-inflammatory functions are linked to the stimulation of other cytokines, such as IL-5 and IL-8, and their very synthesis can be induced by cytokines such as TNF, Interferon, bacterial endotoxins, viruses and different types of other antigens. It is involved in septic shock but it should also be noted that recent studies have demonstrated that IL-1 is capable of activating the expression of some oncogenes and consequently of participating in the pathogenesis of neoplasias. Combined with other cytokines, IL-1 therefore represents one of the major mediators of inflammatory processes: it stimulates T-cells in fact to produce IL-2 and B-cells to produce immunoglobulins. It is also involved in the pathogenesis of rheumatoid arthritis and arthrosis: high quantities of IL-1 have in fact been found in the synovial fluid of patients affected by rheumatoid arthritis and/or osteoarthritis. It is also active in numerous pathologies of a prevalently cutaneous nature, such as dermatitis in general, atopic dermatitis and psoriasis. Finally, it participates in the establishment of vascular damage such as venous thrombosis and is present in all vessels with pathologies of the arterio/arteriosclerotic type. Receptor antagonists are cur-

rently already in clinical use (and also being experimented) for this cytokine, as blockage of the receptor is proving to be an effective way of treating these pathologies in which IL-1 is among the protagonists.

5 TNF: The necrosis factor is part of the group of cytokines which promotes the acute systemic inflammation phase. TNF is therefore involved in an extremely wide number of processes such as cell proliferation, differentiation and apoptosis, carcinogenesis and viral replica-
10 tion.

 It is mostly produced by macrophages and by a series of other cell types including mastocytes, lymphoid cells, muscular and endothelial cells, fibroblasts and nerve cells. Its synthesis can be stimulated by bacterial en-
15 dotoxins, other cytokines such as IL-2, Interferon and IL-1, and it can be inhibited by steroids.

 By acting on numerous organs and systems, generally together with other cytokines, it participates in the establishment and regulation of many pathogenetic processes:
20 esses:

 - it modulates the expression of many proteins and important cytokines, such as IL-1 and IL-6, thus resulting involved in cutaneous pathologies such as vitiligo, eczema, psoriasis and
25 dermatitis in general;

- it stimulates the synthesis of collagenase in the synoviocytes and for this reason, large quantities of TNF have been found in the synovial fluids of patients suffering from arthritis and rheumatoid arthritis;
- it activates the osteoclasts and therefore induces re-absorption of the bone, (osteoporosis);
- it strongly attracts neutrophils and helps them attach themselves to the endothelial cells to extravasate;
- it stimulates the macrophagic production of molecules with an oxidizing action;
- it is involved in particular pathologies of the Cardio-circulatory System participating in the formation of venous thrombosis, in the pathogenesis of arteriosclerosis and vasculitis;

The TNF is capable of binding itself to two receptors, TNF-R1 (receptor for TNF type 1) and TNF-R2 (receptor for TNF type 2), which are expressed in all somatic cells excluding erythrocytes. In short, the TNF promotes the inflammatory response both systemic and cutaneous, which in turn triggers numerous pathologies also of an auto-immune nature, such as rheumatoid arthritis, Crohn's disease, psoriasis and asthma. Scientific research has so

far tried to perfect "biological" drugs (such as, for example, monoclonal antibodies) which inhibit the synthesis of TNF and/or block its receptor.

IL-2: this is a highly pro-inflammatory, atherogenic cytokine, mainly produced by lymphocytes T, whose synthesis is inhibited by steroids and cyclosporines. IL-2 has a central role in regulating the immunological response: it stimulates in fact the synthesis of IFN in the peripheral leukocytes and induces the production of IL-1 and TNF. IL-2 can also damage the hematoencephalic barrier and integrity of the endothelium of the cerebral vessels, causing neuropsychiatric disorders such as disorientation and depression.

There are consequently numerous pathologies which have been associated with an aberrant production of IL-2, such as Hodgkin's lymphoma, multiple sclerosis, rheumatoid arthritis and Lupus erythematosus.

IL-6: produced by many cell types above all I.S., with TNF it is amongst the most important members of the group of chemical mediators of the acute phase of the inflammatory process, and is therefore involved in pathologies with a strong inflammatory component, such as asthma (where it participates in the emergence and maintenance of the inflammatory process), chronic intestinal inflammation (*Crohn's disease*), rheumatoid arthritis and ar-

throsis. As previously affirmed, in fact, cytokines such as TNF, IL-1 and IL-6 have proved to be greatly involved in the degenerative articular osteoarthritis process as they have a primary role in regulating the expression of metalloproteases (responsible for cartilage degradation), in the production of prostaglandins and in osteoclastic activation and, for this reason, high cytokine levels have been registered in the synovial fluids of patients suffering from arthritis and rheumatoid arthritis (R.A.). These discoveries have stimulated the use of inhibitors in the above interleukins and/or receptor antagonists as a new treatment strategy of the arthritis pathology.

Finally, recent studies have connected cancer with longevity and revealed how some tumors are influenced by the kind of/quantitative situation of the cytokine proteins of the patient: in short, recent evidence has linked a low production profile of IL-10 and high secretion of IL-6 to a deterioration in the clinical survival of patients affected by tumoral pathologies, whereas a genotype capable of producing and maintaining high levels of IL-10 can facilitate survival (Caruso C. et al., Ann N.Y. Acad. Sci., 2004, 1028:1-13).

IL-7: a cytokine mainly produced by stromal cells of bone marrow, it is also secreted by the thymus and keratinocytes. IL-7 induces the synthesis of inflammatory cyto-

kines such as IL-1, IL-6 and TNF, thus participating in the pathogenesis of some skin diseases (such as psoriasis and cutaneous lymphoma) and the osteoarticular system, high levels of IL-7 have in fact been found in patients
5 suffering from R.A.

IL-12: this protein also plays a central role in regulating the functions of the I.S. It acts in fact on the differentiation of the lymphocytes, it induces the synthesis of Interferon and TNF, and its production can be inhibited by IL-10. The over-production of this protein enters in the pathogenesis of diseases of an auto-immune nature such as colitis, arthritis, insulin-dependent diabetes, encephalomyelitis, psoriasis and multiple sclerosis (Brahmachari S. et al., Minerva Med., 2008, 99(2):105-
10 118).
15 118).

IL-10: mainly produced by lymphocytes, it is a cytokine of an anti-inflammatory nature, capable of inhibiting the synthesis of IL-2 and Interferon produced by lymphocytes T. The anti-inflammatory action of IL-10 is also revealed in the capacity of inhibiting the synthesis of IL-1, IL-
20 6, IL-8, IL-12 and TNF in the macrophages stimulated with bacterial endotoxins. IL-10 deficiencies are associated with pathologies such as diabetes mellitus and chronic intestinal inflammations, such as Crohn's disease. Recent
25 evidence has led IL-10 to also be experimented as a new

therapeutic approach for the treatment of Lupus erythematosus. . Low IL-10 levels have been observed in cutaneous tissues of patients suffering of pathologies such as vitiligo, psoriasis, eczema and dermatitis. It should be
5 noted that both corticosteroids and cyclosporine increase the production and/or release of this interleukin from the relative competent cells during conventional immunosuppression therapy for the treatment of inflammations and organ rejection (Zhou X. Et al., Current Drug Tar-
10 gets-Immune, Endocrine & Metabolic Disorders, 2005, 5(465475). Experimental data have also demonstrated its effectiveness in reducing the release of prostaglandins and cyclo-oxygenase induced *in vitro* by TNF on human synoviocytes, thus indicating the capacity of IL-10 of
15 reducing inflammatory processes which involve articulations affected by osteoarthrosic degeneration (Alaaeddine N. et al., Arthritis & Rheumatism, 1999, 42:710-718). Recent studies have confirmed its therapeutic effectiveness towards the asthma pathology in experimental animal models of bronchial hyper-reactivity, showing how this cyto-
20 kine has a high therapeutic potentiality in reducing the inflammation which characterizes the air passages of asthmatic patients, in which high concentrations of TNF, IL-1, IL-5, IL-6 and IL-8 have been found in the bron-
25 chial-washing liquid and/or on a serum level and/or tis-

sue level (Stankiewicz W. et al., Mediators of Inflammation, 2002, 11:307-312). For this interleukin, the important role of regulator cytokine of the maintenance of immunological homeostasis, has therefore been assumed.

5 Asthma can be an extremely debilitating disease of which approximately 200 million people in the world suffer, with over 5,000 deaths per year. It is a pathology which is based on a distorted response of the I.S. to environmental factors, consequently linked to an exacerbated
10 ed production of pro-inflammatory cytokines for the growth and differentiation of mast cells and eosinophils with other types of cells of the I.S.. The causes of this out-of-balance activity of the immune system are still not completely known, there are however genetic, environ-
15 mental, viral and also nutritional factors which contribute in different ways to the development of this pathology. Consequently, finding an effective therapy (systemic and/or local therapy) for its prevention and/or treatment which allows the suspension or reduction of the use of
20 steroids (conventional treatment therapy), could represent a valid solution for both the more serious forms (as it would in any case enable a reduction in the use of steroids) and for less serious cases, as the suspension of the steroid therapy could be total.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a graph showing gene expression of IL-10 in human synoviocytes treated with HA vs HAS1 (0.5 mg/ml) and HAS3 (0.1 mg/ml);

5 Figure 2 is a graph showing gene expression of IL-12 in human synoviocytes treated with HA vs HAS1 and HAS3 (0.5 mg/ml);

Figure 3 is a graph showing HAS1 and HAS3: binding inhibition % of TNF in U937 human cells;

10 Figure 4 is a graph showing HAS3: binding inhibition % of IL-1 in mouse 3T3 cells;

Figure 5 is a graph showing HAS3: binding inhibition % of IL-6 in U266 human cells;

Figure 6 is a graph showing synthesis of IL-2
15 in human PBMC treated with HA vs HAS1 (0.1 mg/ml) and HAS3 (1 mg/ml);

Figure 7 is a graph showing synthesis of IL-7 in human PBMC treated with HA vs HAS1 (1 mg/ml) and HAS3 (1 mg/ml);

20 Figure 8 is a graph showing synthesis of IL-12 in human PBMC treated with HA vs HAS1 (1 mg/ml) and HAS3 (1 mg/ml);

Figure 9 is a graph showing synthesis of IL-10 in human PBMC treated with HA vs HAS1 (0.1 mg/ml) and HAS3
25 (0.1 mg/ml);

Figure 10 is a graph showing permeation of HA and sulfated HA with a low (20 KD) and medium (200 KD) MW;

Figure 11 is a graph showing skin absorption in 24h of diclofenac for formulations containing HAS vs HA and
5 control;

Figure 12 is a graph showing skin hydration with HAS3 vs HA and vs control base cream; and

Figure 13 is a graph showing skin roughness with HAS3 vs HA and vs control base cream.

10 DETAILED DESCRIPTION OF THE INVENTION

The invention is directed to a new and surprising topic use of HAS as regulator agent of the cytokine activity, as the Applicant has discovered its exclusive capacity in modulating the activity of particular cytokines, it has studied its action mechanism and revealed the substantial difference between the various types of sulfated products known in the state of the art, but above all the Applicant has discovered an unexpected activity vs different types and strains of Herpes virus, Cytomegalovirus and the virus of vesicular stomatitis. Finally, a further object of the present invention relates to the use of HAS as promoter of the skin absorption of drugs of a prevalently anti-inflammatory nature, as a fibrinolytic agent and also as a highly hydrating agent for the treatment therapy of all skin pathologies characterized by dryness, irritation and redness, inflammation and desquamation.

The sulfated hyaluronic acid suitable for the purposes of the present invention is prepared according to the process described in EP 702699 B1: the sulfation is effected by means of the complex SO₃-pyridine and involves the alcohol hydroxyls present in the polysaccharide chain starting from a HA deriving from any source, for example, by extraction from cockscombs, either fermentatively or biotechnologically, and having a molecular

weight ranging from 400 to 3×10^6 Da, in particular from 1×10^4 Da to 1×10^6 Da, even more in particular from 10,000 to 50,000 Da, 150,000 to 250,000 Da and 500,000 to 750,000 Da.

5 The derivative obtained maintains all the physical characteristics of the starting polymer unaltered, in particular the molecular weight of the starting HA is not reduced by the sulfation process thus allowing all the physico-chemical characteristics of the starting polysac-
10 charide to be maintained. The sulfation involves the various hydroxyl groups of the disaccharide unit and it is therefore possible to obtain different sulfation degrees, from 0.5 to 3.5 (intended as the number of sulfate groups per disaccharide unit), by varying the quantity of
15 SO_3 -pyridine introduced as known in the state of the art.

 The derivative used in all the experimentations effected generally has sulfation degree 1 or degree 3 and is defined hereafter as HAS1 and HAS3. All the free carboxyl groups of the HA can be salified with cations of an
20 organic and/or inorganic origin.

 Both degrees of HAS are soluble in water and they can also be sterilized with the normal techniques known to experts in the field, even if sterilization using an autoclave is preferable.

25 The Applicant describes and claims the new use of

HAS for the preparation of a medicine for topic use:

- for the prevention and/or treatment of skin pathologies associated with immune deficiency and, in particular, deficiency of IL-10, such as vitiligo, eczema, psoriasis and dermatitis in general, stimulating the synthesis of anti-inflammatory cytokines;
- for the prevention and/or topic treatment of asthma, associated with the activation of IL-1, IL-6 and TNF by inhalation;
- for the prevention and/or treatment of skin pathologies associated with damage to the endothelium and/or wall of blood vessels due, for example, to traumas, vascular hemorrhages of a surface nature and/or of medium depth with the consequent formation of clots and edemas;
- for the prevention and/or cutaneous treatment of skin diseases associated with the increase/activation of IL-1, IL-2, IL-6, IL-7, IL-8, IL-12 and TNF such as, for example, dermatitis, atopic dermatitis, psoriasis, vitiligo, photodermatitis, urticaria, all skin irritations (also gingival) and eczema;
- for the prevention and/or treatment of diseases of an autoimmune nature such as psoriasis, asthma and skin manifestation of systemic (LES) and discoid Lupus erythematosus;
- for the prevention and/or topic treatment of skin neo-

plasias such as, for example, basalioma, Kaposi sarcoma, squamous cell carcinoma, cutaneous lymphoma, mycosis fungoides and actinic keratosis;

- for the prevention and/or topic treatment of vascular pathologies such as, for example, vasculitis and scleroderma, associated with the activation of TNF, IL-1 and IL-6;

The Applicant has in fact also demonstrated, in the experimentations described hereunder, that:

- 10 • HAS is capable of both stimulating the production of new mRNA and the protein synthesis of cytokines of an anti-inflammatory nature (such as, for example, IL-10), thus increasing the immune defense capacity of the cells and consequently the whole organism. The anti-inflammatory action of the above cytokines is revealed
15 in the capacity of inhibiting the synthesis of IL-1, IL-6, IL-8, IL-12 and TNF, all highly pro-inflammatory proteins involved in numerous skin pathologies.
- HAS is effective in both diminishing the synthesis of new mRNA and in significantly reducing the protein synthesis of IL-2, IL-7 and IL-12, in situations in which
20 an immune response is not solicited and in particular events of inflammatory stress in which the cells respond by producing a cytokine cascade: especially in
25 this case, the data presented reveal the greater effect

of HAS.

- HAS is effective in inhibiting the binding of TNF, IL-1 and IL-6 to their receptor. These results are of fundamental importance as they prove that the behaviour of the sulfated product is completely analogous to that of monoclonal antibodies specific for the receptors of the above pro-inflammatory proteins, capable therefore of blocking their function, but at the same time not having this antibody specificity. This receptor blockage represents the most effective way of antagonizing the pro-inflammatory and tumoral effects of the TNF, IL-1 and IL-6 factor, thus opening up new horizons for clinical experimentation, allowing the perfectioning of new therapeutic approaches in the treatment and/or prevention of an extremely large number of pathologies, considering the role that TNF, IL-1 and IL-6 play in the emergence and progression of numerous systemic and skin diseases.

The Applicant also describes and claims the new use of HAS for the preparation of a medicine for topic use:

- for the prevention and/or treatment of Herpes Simplex labialis and Herpes genitalis;
- for the prevention and/or treatment of the virus of vesicular stomatitis;
- for the prevention and/or treatment of Cytomegalovirus.

The Applicant has in fact demonstrated, in the experimentations described hereunder, the powerful antiviral action of HAS vs different types of virus:

- Experimental data prove the antiviral action of HAS1 and HAS3 vs Herpes Simplex virus 1 and 2 and vs the virus of vesicular stomatitis (VSV). The first form, extremely widespread, is responsible for the appearance of characteristic febrile vesicles which normally affect the facial cutis (lips, nostrils); it is also called herpes simplex labialis. The infection caused by herpes *labialis* can easily reappear as the virus survives inside the cells and is not even eliminated with the use of effective drugs. The second form is a genital infection, also known as herpes genitalis. Both are caught by physical or sexual contact. Due to the location of the virions in the nervous ganglia, where they can remain quiescent for a long period of time, the herpetic infection has recurring characteristics in correspondence with stressing events of the immune system and usually reappears in the primary site. The virus of vesicular stomatitis is an RNA-virus, it strikes mammals and is used in the laboratory for studying the development of the life-cycle of the RNA-virus. A comparison between HA-NS1 and HAS1 shows once again that not all sulfated hyaluronic acids are equivalent, as

HA-NS1 has proved not to be active at all, whereas both HAS 1 and 3 show a very strong antiviral activity vs Herpes Simplex and also vs VSV. None of the samples tested prove to be cytotoxic towards the host cell, the
5 minimum cytotoxic concentration obtained, in fact, is equal to that of reference drugs normally used in clinical practice for the treatment of Herpes, and on an average has proved to be 100 times higher than that revealed active in the inhibition of viral replication.

10 • Experimental data obtained for both HAS1 and HAS3 have revealed a clear and significant antiviral result vs Cytomegalovirus: this is a particular type of virus which enters some types of cells of our organism in which it reproduces itself parasitically causing their
15 death. It belongs to the same family as *herpes labialis* and *herpes genitalis*, chickenpox and infective mononucleosis. Epithelial cells, mucous membranes, lymphonodes are the site of multiple primary infection. It remains in latent form for life in the peripheral
20 blood, in the epithelium of the renal tubules and in the epithelium of the salivary glands. Serious forms are found in immunocompromised subjects (such as those affected by AIDS and transplant subjects in immunosuppressive therapy). The treatment therapy consists in
25 the administration of drugs such as ganciclovir,

valganciclovir and foscarnet (inhibitors of the synthesis of viral DNA). Also in this case, HA-NS1 has proved to be non-active in inhibiting the proliferation of the virus confirming the absolute diversity, as antiviral capacity, between the two types of sulfated products.

The present invention also relates to the use of HAS as a fibrinolytic agent for the degradation of fibrin clots which are formed on a cutaneous level (surface and/or in depth) following the breakage of the endothelium and/or wall of the capillaries and/or small vessels, due to mechanical traumas and/or hemorrhages of a medium/small entity.

In the experimentations described hereunder, the Applicant has in fact demonstrated:

- that HAS is effective as Plasmin in the fibrinolysis/debridement of clots and thrombi. **Plasmin** is an important enzyme belonging to the group of hydrolases capable of degrading many proteins of blood plasma, and in particular the fibrin in thrombi and clots. The degradation of the fibrin is called fibrinolysis. A plasmin deficiency can lead to thrombosis, as the thrombi are not adequately degraded. The substantial difference between the anti-coagulating process and fibrinolytic process should be noted: in the former case the anti-coagulating agent must prevent the formation of coagulum, in the lat-

ter case, the fibrinolytic agent, on the other hand, must intervene in a situation in which the coagulum is already present, and must therefore be degraded for its total elimination.

5 In addition, the present invention relates to the new use of HAS as a skin absorption promoter of drugs such as, for example, those of an anti-inflammatory nature and, finally, as a highly dehydrating agent for the treatment therapy of all skin pathologies characterized
10 by dryness, lichenification, irritation, pruritus and redness, inflammation and desquamation.

The Applicant has in fact demonstrated that:

- the sulfation of hyaluronic acid substantially increases skin absorption, consequently,
- 15 • the hydrating power of HAS has proved to be significantly higher than that of non-sulfated HA and therefore causes an important decrease in the roughness of treated skin surfaces with respect to HA and topic control formulations, thus revealing its capacity of effectively
20 treating and protecting skin surfaces characterized by dryness, irritation, lichenification, pruritus and redness, inflammation and desquamation with all the other skin pathologies which make the skin more sensitive to external agents;
- 25 • HAS is a powerful and effective skin absorption pro-

moter of drugs. The capacity of HAS of penetrating the skin thickness so efficiently is the scientific basis on which this surprising and unexpected new property is founded, which enables its formulation with pharmacological agents of a varying nature, such as for example, of the non-steroid anti-inflammatory (in particular diclofenac, ketoprofene and ibuprofene) or steroid type, hormones, vaso-dilators, cholinergic agents, antibiotics and others, formulated in various forms, preferably as gels, creams or patches for a dermal and/or transdermal absorption.

Finally, the Applicant describes the preparation of various topic pharmaceutical formulations/compositions containing HAS as sole active principle, or in association with other pharmacologically and/or biologically active agents such as, for example, steroids, hormones, proteins, trophic factors, vitamins, non-steroid anti-inflammatory drugs (FANS) such as, for example diclofenac, ketoprofene or ibuprofene or salts thereof, chemotherapy drugs for topic use, antibiotics, antiviral agents, local anesthetics, anticoagulants and/or fibrinolytic agents, and/or enzymes such as, for example, collagenase and/or hyaluronidase and/or other proteases; it can be formulated with polymers such as hyaluronic acid and its derivatives, carboxymethylcellulose (CMC) and/or

other polymers of a natural (such as collagen) or synthetic nature.

The pharmaceutical composition in question can be formulated as an ointment, lipogel, hydrogel, lipstick, cream, vaginal ovules and bougies, foam, mucosal gel, ophthalmic preparations, vaginal douches, mouthwash, patches for dermal and/or transdermal absorption, especially of FANS and hormones, solutions, it can therefore be administered by topic application or by inhalation for the treatment of pathologies of the respiratory system such as asthma, for example.

Particular attention is paid to compositions containing enzymes such as hyaluronidase in the formulation of a medicine for the treatment of skin hematomas, and those containing non-steroid anti-inflammatory drugs or hormones, in the form of gels, creams and patches for the dermal and/or transdermal absorption of the drug.

In one particular embodiment the invention provides sulphated hyaluronic acid for topical use as a hydrating agent for the treatment therapy of skin pathologies characterized by dryness, lichenification, irritation, pruritus and redness, inflammation and desquamation, said sulphated hyaluronic acid (HA) being obtained by a sulfation

involving the alcohol hydroxyls present in the HA polysaccharide chain, starting from a hyaluronic acid (HA) having a molecular weight ranging from 10,000 to 50,000 Da, 150,000 to 250,000 Da or 500,000 to 750,000 Da and a
5 sulphation degree equal to 1 or 3.

Some examples of the preparation of HAS degree 1 and 3, pharmaceutical formulations containing it, are provided for purely descriptive and non-limiting purposes, together with the results obtained by
10 experimentation *in vitro*.

Example 1

Preparation of the tetrabutylammonium salt of hyaluronic acid (HA) having an average molecular weight equal to 200 KD (ranging from 150,000 to 250,000 Da)

15 5.00 g of hyaluronic acid sodium salt of a fermentative origin (200 KD) are dissolved in 250 ml of water and the resulting solution is percolated through a glass column pre-filled with 100 cm³ of Dowex™ resin in the form of tetrabutylammonium (TBA). The eluted solution of
20 HA-TBA salt is collected and freeze-dried. 7.50 g of product are obtained.

Example 2

Synthesis of sulfated HA starting from HA having an average molecular weight of 200 KD and a sulfation degree
25 equal to 3 sulfate groups per repetitive unit

Method A

10.0 g of the TBA salt of hyaluronic acid having an average molecular weight of 200 KD prepared according to Example 1, are dissolved in 300 ml of dimethylsulfoxide(DMSO); 26.0 g of the complex SO₃-
5 pyridine (sulfur trioxide and pyridine, hereafter abbreviated as PySO₃) are dispersed in 150 ml of DMSO, and then added to the solution of HA. After 20 hours under mechanical stirring at a temperature of 21°C, the reaction is interrupted by
10 adding 0.1 volumes of water; the raw reaction product is isolated by precipitation after the addition of 2 volumes of ethanol. The solid obtained is dispersed in 150 ml of water and the pH brought to neutrality with NaOH 1 M. The

mixture is exhaustively dialyzed against water through a membrane with a cut-off of 12-14,000 Da. The dialyzed product is subjected to freeze-drying. 9.7 g of product are obtained with a sulfation degree equal to 3 sulfate groups per repetitive unit (yield = 88%).

Method B

32.0 g of the TBA salt of hyaluronic acid having an average molecular weight of 200 KD prepared according to Example 1, are dissolved in 900 ml of N-Methyl-Pyrrolidone (NMP); 100 g of PySO_3 are dispersed in 600 ml of NMP, and then added to the solution of HA. After 20 hours under mechanical stirring at a temperature of $21 \pm 1^\circ\text{C}$, the reaction is interrupted by adding 0.5 volumes of water. The pH initially lower than 2.5, is brought to neutrality by the addition of a mole of NaOH (in solution). The reaction raw product is isolated by precipitation by the addition of 2.5 volumes of methanol and washed with 2 volumes of a methanol/water mixture 8/2. The solid is re-dissolved and exhaustively dialyzed against water using a membrane with a cut-off of 12-14,000 Da. 30.4 g of product are obtained with a sulfation degree equal to 3 sulfate groups per repetitive unit (yield = 86%).

Example 3

Synthesis of sulfated HA starting from HA having an aver-

age molecular weight of 200 KD and a sulfation degree equal to 1 sulfate group per repetitive unit

Using the procedure illustrated in Example 1, 10.0 g of TBA salt of HA are prepared, which are dissolved in 350 ml of DMSO. 10.0 g of the complex PySO_3 are dispersed in 100 ml of DMSO, and then added to the solution of HA. After 20 hours under mechanical stirring at a temperature of 21°C, the reaction is interrupted by adding 0.1 volumes of water; the raw reaction product is isolated by precipitation after the addition of 2.5 volumes of ethanol. The solid obtained is dispersed in 150 ml of water and the pH brought to neutrality with NaOH 1 moles/l. The mixture is exhaustively dialyzed against water through a membrane with a cut-off of 12-14,000 Da. The dialyzed product is subjected to freeze-drying. 7.54 g of product are obtained with a sulfation degree equal to 1.0 sulfate group per repetitive unit (yield = 93%).

Example 4

Synthesis of sulfated HA starting from HA having a low molecular weight (average MW of 10 KD, ranging from 5,000 to 30,000 Da) and a sulfation degree equal to 3 sulfate groups per repetitive unit

Using the procedure illustrated in Example 1, 12.4 g of TBA salt of hyaluronic acid of low-molecular weight are prepared, which are dissolved in 300 ml of NMP. 40 g

of PySO_3 are dispersed in 100 ml of NMP, and then added to the solution of HA. After 20 hours under mechanical stirring at a temperature of 21°C , the reaction is interrupted by adding 0.5 volumes of water. The pH initially
5 lower than 2.5, is brought to neutrality by the addition of a mole of NaOH 4M. The reaction raw product is isolated by precipitation by the addition of 2.5 volumes of methanol and washed with 2 volumes of a methanol/water mixture 8/2. The solid is re-dissolved and exhaustively
10 dialyzed against water using a membrane with a cut-off of 3,500 Da. 12.0 g of product are obtained with a sulfation degree equal to 3.0 sulfate groups per repetitive unit (yield = 85%).

Example 5

15 Synthesis of sulfated HA starting from HA having a low molecular weight and a sulfation degree equal to 1 sulfate group per repetitive unit

Using the procedure illustrated in Example 1, 12.4 g of TBA salt of HA are dissolved in 300 ml of DMSO. 16.0 g
20 of PySO_3 are dispersed in 100 ml of DMSO and then added to the solution of HA. After 20 hours under mechanical stirring at a temperature of 21°C , the reaction is interrupted by adding 0.1 volumes of water; the reaction raw product is isolated by precipitation after the addition
25 of 2.5 volumes of ethanol. The solid obtained is dis-

persed in 150 ml of water and the pH brought to neutrality with NaOH 1 moles/l. The mixture is exhaustively dialyzed against water through a membrane with a cut-off of 3,500 Da. The dialyzed product is subjected to freeze-drying. 9.04 g of product are obtained with a sulfation degree equal to 1.0 sulfate group per repetitive unit (yield = 90%).

Example 6

Synthesis of sulfated HA starting from HA having a molecular weight within the range of 500-730 KD and a sulfation degree equal to 3 sulfate groups per repetitive unit

21.0 g of hyaluronic acid sodium salt of an extractive origin (500-730 KD) are dissolved in 1.5 l of water and the resulting solution is percolated through a glass column pre-filled with 450 cm³ of Dowex resin in the form of TBA. The eluted solution of HA-TBA salt is collected and freeze-dried. 32.0 g of product are obtained, which are dissolved in 1.35 l of NMP; 100 g of PySO₃ are dispersed in 650 ml of NMP, and then added to the solution of HA. After 20 hours under mechanical stirring at a temperature of $23 \pm 1^{\circ}\text{C}$, the reaction is interrupted by adding 0.5 volumes of water. The pH initially lower than 2.5, is brought to neutrality by the addition of NaOH (in solution at a concentration of 4 moles/l). The reaction

raw product is isolated by precipitation by the addition of 2.5 volumes of methanol and washed with 3.5 volumes of a methanol/water mixture 8/2. The solid is re-dissolved and exhaustively dialyzed against water using a membrane
5 with a cut-off of 12-14,000 Da. 30.3 g of product are obtained with a sulfation degree equal to 3 sulfate groups per repetitive unit (yield = 83%).

Example 7

Synthesis of sulfated HA starting from HA having a mo-
10 lecular weight of 500-730 KD and a sulfation degree equal
to 1 sulfate group per repetitive unit

21.0 g of hyaluronic acid sodium salt of an extractive origin (500-730 KD) are dissolved in 1.5 l of water and the resulting solution is percolated through a glass
15 column pre-filled with 450 cm³ of Dowex resin in the form of TBA. The eluted solution of HA-TBA salt is collected and freeze-dried. 32.0 g of product are obtained, which are dissolved in 1.65 l of NMP; 40 g of PySO₃ are dispersed in 350 ml of NMP, and then added to the solution
20 of HA. After 20 hours under mechanical stirring at a temperature of 25 ± 1°C, the reaction is interrupted by adding 0.5 volumes of water. The pH initially lower than 2.5, is brought to neutrality by the addition of NaOH (in solution at a concentration of 4 moles/l). The reaction
25 raw product is isolated by precipitation by the addition

of 3.5 volumes of methanol and washed with 3.5 volumes of a methanol/water mixture 8/2. The solid is re-dissolved and exhaustively dialyzed against water using a membrane with a cut-off of 12-14,000 Da. 22.5 g of product are obtained with a sulfation degree equal to 1.0 sulfate group per repetitive unit (yield = 87%).

Example 8

Evaluation of the regulatory effect of HAS degree 1 and degree 3 on the gene expression of IL-10 and IL-12

Human synoviocytes previously expanded in vitro and maintained in a culture at 37°C with a medium of DMEM containing 10% of FCS, were seeded at a concentration of 20,000 cells per well (synoviocytes are cells capable of producing various types of cytokines, and are therefore normally used for this type of experimental test). Sulfated HA degree 1 (HAS1) and degree 3 (HAS3) prepared as described in Examples 1-3, were then added to the culture medium at concentrations of 0.1 and 0.5 mg/ml (for both samples), whereas the control treatment is represented by non-sulfated HA having an average molecular weight (MW) of 200 KD. After 3 days of treatment, the PCR Real Time was effected to evaluate the gene expression of IL-10 and IL-12: the cell RNA was extracted using the "Trizol" method, following the indications of the supplier (TRIZOL reagent, LIFE Technologies, GIBCO BRL). In short, the

cells were lysed by the addition of 1.0 ml of Trizol and the total RNA was quantified by measuring its absorbance at 260 nm. The appropriate *primers* were selected for each gene to be amplified, using the software Primer3™ (Roche
5 Molecular Diagnostics, Pleasanton, CA, USA). The gene expression was evaluated by means of *PCR Real Time* effected with a Rotor-gene™ TM5500 (Corbett research, Sydney, Australia). The PCR reactions were effected using *primers* at 300 nm and SYBR Green (Invitroge, Carlsbad,
10 CA, USA) at 40 cycles of 15s at 95°C, and 1 min. at 60°C. The value of "*Fluorescence thresholds (Ct)*" was automatically determined by the software, evaluating an amplification coefficient for the genes studied between 92 and 110%. For each sample of cDNA, the gene expression
15 value was expressed in terms of the ratio between the ct of the *house keeping* gene (i.e. the gene for the beta-Actin protein which represents the control gene as it is present in every cell and is not subjected to the influence of HAS) and the ct of the gene of interest
20 (i.e. the gene for IL-10 and IL-12), consequently the house keeping ct/gene ct value is indicated in the axis of the ordinates, which therefore indicates the quantity of mRNA expressed by the gene which is being studied. The results obtained are expressed in Figure 1 and 2:

25 Fig. 1: the treatment of human synoviocytes with HAS1 and

HAS3 caused a significant increase in the gene expression of the cytokine IL-10 vs the control treated with non-sulfated HA.

Fig. 2:

5 Also in this experiment, both sulfation degrees (degree 1 and degree 3) of HAS proved to be capable of significantly reducing the gene expression of IL-12, halving the synthesis of its mRNA vs the control treated with non-sulfated HA. The sulfated hyaluronic acid therefore
10 proved to be:

- capable of stimulating the production of new mRNA for the synthesis of anti-inflammatory cytokines, thus increasing the defense capacity of the cell and consequently of the whole organism, vs those pathologies
15 previously described in which IL-10 proved to be of fundamental importance for the resolution and/or improvement of diseases such as asthma, vitiligo and all inflammations in which IL-10 is involved.
- effective in diminishing the synthesis of new mRNA of
20 the highly pro-inflammatory cytokine IL-12, proving to be a valid anti-inflammatory agent capable of intervening on the expression of proteins involved in the pathogenesis of invalidating diseases such as psoriasis and all those previously described.

25 **Example 9**

Inhibition of the binding of TNF to its receptor expressed in monocyte lines: evaluation of the effectiveness of HAS degree 1 and degree 3 at different MW values

These experiments were effected to evaluate the effectiveness of the samples tested (prepared according to Examples 1-4) on the capacity of inhibiting the binding of TNF to its receptor expressed by cells of the I.S. normally used *in vitro* for this type of experiment, carried out with iodinated cytokine components for an evaluation in *Radioligand Binding assays*.

The experimental procedure was effected as described in Baglioni C. et al., J Biol Chem, 1985, 260:13395-13397.

In short, the line of human histiocytes of the lymphoma U937 was used, with characteristics of monocytes sensitive to the cytotoxic activity of TNF, expressing its relative receptor. The cells were initially incubated with ^{125}I -TNF 0,028nM (carried in water) contemporaneously with the samples to be analyzed (at a concentration of 1 mg/ml which proved to be the lowest concentration which causes the maximum inhibition), in an incubation buffer consisting of 50 mM Tris-HCL pH 7,4, 0,5 mM EDTA, at 4°C for 3 hours.

At the end of the incubation, the cells were centrifuged with dibutylphthalate/dinonylphthalate 2/1 and the

pellet obtained was counted in a γ -counter.

The results obtained are expressed in Figure 3:

The results obtained show the effectiveness of HAS in totally (100%) inhibiting the binding of TNF to its
5 receptor, for both degree 1 and degree 3, with a medium and low MW. These results are of fundamental importance as they prove that the behaviour of the sulfated product is completely analogous to that of a monoclonal antibody specific for the TNF receptor, capable therefore of
10 blocking its function. This receptor blockage consequently represents the most effective way of antagonizing the pro-inflammatory and tumoral effects of the TNF factor.

Example 10

15 Inhibition of the binding of the cytokine IL-1 to its receptor expressed in fibroblast lines: evaluation of the effectiveness of HAS degree 3 at different MW values

These experiments were effected to evaluate the effectiveness of the samples tested (prepared according to
20 Examples 1-3 and 4) on the capacity of inhibiting the binding of IL-1 to its receptor expressed by 3T3 cells of mouse, normally used *in vitro* for this type of experiment, carried out with iodinated cytokine components for an evaluation in *Radioligand Binding assays*.

25 The experimental procedure was effected as described

in Chin J et al., J Exp Med, 1987, 165:70-86.

In short, the line of murine fibroblasts 3T3 was used, sensitive to the cytotoxic activity of IL-1, expressing its relative receptor. The cells were initially
5 incubated with ^{125}I -IL-1 10 pM (carried in water) contemporaneously with the samples to be analyzed (at a concentration of 1 mg/ml which proved to be the lowest concentration which causes the maximum inhibition), in an incubation buffer consisting of RPMI 1640 containing 20 mM
10 HEPES pH 7.2 and 1% BSA, at 37°C for 2 hours. At the end of the incubation, the cells were washed with phosphate buffer, then dissolved in 2.5 M of NaOH and counted in a γ -counter.

The results obtained are expressed in Figure 4:

15 The results obtained show the effectiveness of HAS (with both medium and low MW) in inhibiting the binding of IL-1 to its receptor at 30%. These results are extremely significant as they prove that the behaviour of the sulfated product is completely analogous to that of a
20 monoclonal antibody specific for the receptor of the cytokine in question, capable therefore of blocking its function. This receptor blockage represents the most effective way of antagonizing the pro-inflammatory and tumoral effects of IL-1, as previously described.

25 Example 11

Inhibition of the binding of the cytokine IL-6 to its receptor expressed in human myeloma cells: evaluation of the effectiveness of HAS degree 3 at different MW values

These experiments were effected to evaluate the effectiveness of the samples tested (prepared according to Examples 1-3 and 4) on the capacity of inhibiting the binding of IL-6 to its receptor expressed in human myeloma U266, normally used *in vitro* for this type of experiment, carried out with iodinated cytokine components for an evaluation in *Radioligand Binding assays*.

The experimental procedure was effected as described in Taga T. et al., *J Exp Med*, 1987, 166:967-981.

In short, the line of human myeloma U266 was used, sensitive to the cytotoxic activity of IL-6, expressing its relative receptor. The cells were initially incubated with ^{125}I -IL-6 0,08nM (carried in water) contemporaneously with the samples to be analyzed (at a concentration of 1 mg/ml which proved to be the lowest concentration which causes the maximum inhibition), in an incubation buffer consisting of RPMI 1640 containing 25 mM HEPES pH 7.1 and 10% BSA, at 4°C for 16 hours. At the end of the incubation, the cells were washed with phosphate buffer, centrifuged at 9,000 rpm and the pellet counted in a γ -counter.

The results obtained are expressed in Figure 5:

The results obtained show the effectiveness of HAS, with both medium and low MW, in totally (100%) inhibiting the binding of IL-6 to its receptor. These results consequently prove that the behaviour of the sulfated product, also in this case, is completely analogous to that of a monoclonal antibody specific for the receptor of the cytokine in question, capable therefore of blocking its function. This receptor blockage represents the most effective way of blocking the pro-inflammatory effects of IL-6.

Example 12

Evaluation of the inhibitory effect of HAS degree 1 and degree 3 on the protein synthesis of the cytokines IL-2, IL-7, IL-10 and IL-12 in human PBMC

For these experimentations, mononucleate cells of human peripheral blood (PBMC) were adopted, deriving from various donors for evaluating the effect of HAS on the production of the cytokines listed above, using:

- non-sulfated HA (average MW: 200 KD),
- HAS1 and HAS3 (prepared as described in Examples 1-3).

The separation of the PBMC (Bøyum A., *Scand J Clin Lab Invest* 21 Suppl, 1968, 97:77-89) was effected using the product Ficoll-Paque PLUS™ (GE Healthcare) and following the protocol indicated by the supplier. At day zero 100,000 cells were plated per well (using plates with 96

wells) in 200 µl of medium RPMI 1640, to which 10% of fetal bovine serum, HEPES 10 mM, Glutamine 2mM, 1% Penicillin-Streptomycin 100 U/ml, had been added. The effect of all the samples was evaluated on non-treated PBMC, or
5 stimulated with Lipopolysaccharide LPS (10 µg/ml) (highly pro-inflammatory) or with Phytohemagglutinin PHA (10 µg/ml) (a substance capable of stimulating the lymphocytes to divide themselves), both agents capable of stimulating the synthesis of cytokines. The cells were
10 treated separately with the three compounds at a concentration of 0.1 mg/ml or 1 mg/ml. After 24 hours of incubation at 37°C (5% CO₂), 100 µl of supernatant were taken from each well in order to analyze the production of IL-2, IL-7, IL-10 and IL-12.

15 The quantification of the inflammation mediators was effected by means of the *SearchLight*[®] technology, using a *Custom Human 9-Plex Array* plate following the protocol indicated by the supplier in the technical card.

The results obtained are expressed in Figures 6-9:

20 These graphs clearly show that HAS degree 1 and degree 3 are capable of significantly reducing the synthesis of IL-2, IL-7 and IL-12 on the part of monocytes, both when the cells are not stimulated and also when, on the contrary, they are stimulated by specific and powerful inflammatory factors and/or mitogens. HAS therefore proves
25

to be a molecule with precise pharmacological characteristics, capable of modulating/regulating the synthesis of cytokines with a marked anti-inflammatory activity, both in situations where an immune response is not stimulated, and in particular inflammatory stress events in which the immune cell responds by producing a cytokine cascade and, above all, in this case, the data presented reveal a greater HAS modulating effect.

Figure 9, on the other hand, confirms the evident stimulus for the production of IL-10 also for cells belonging to the Immune System. It is consequently again confirmed that HAS is capable of modulating the synthesis of cytokines, stimulating those which are anti-inflammatory and inhibiting the synthesis of pro-inflammatory cytokines.

Example 13

Evaluation of the antiviral action of HAS degree 1 and degree 3 vs HA-NS:

Herpes Simplex virus-1, Herpes Simplex virus-2, Vesicular stomatitis virus

The activity of the samples tested was determined by evaluating the inhibition of the cytopathogenicity caused by the virus of Herpes Simplex Virus-1, (HSV-1: KOS, F and McIntyre strain) and by the virus of Herpes Simplex Virus-2, (HSV-2: G, 196 and Lyons strain) in E₆SM fibro-

blast cells which derive from muscular/cutaneous embryonic tissue. Furthermore, the anti-viral activity was tested again vs E₆SM cells infected by the virus of vesicular stomatitis (Vesicular stomatitis virus: VSV).

5 HSV-1 is the virus which preferentially infects the oral mucous, whereas HSV-2 attacks the genital mucous. The experimental procedure was effected as described in Baba M. et al., ANTIMICROB. AGENTS CHEMOTHER., 1988, 32:1742-1745.

10 In short, confluent cell cultures were exposed to infective doses of the viruses listed above in the presence of the samples HS-NS1 (EP0971961), HAS1 and HAS3 prepared as described in Example 1-3. After an incubation period of 1h at 37°C, the culture medium was substituted

15 with fresh medium only containing the samples to be tested. The cytopathogenicity of the virus was tested on the 2nd day of incubation. The measurement of the inhibition of the viral cytopathogenicity was evaluated by determining the inhibition of the synthesis of DNA and RNA

20 in the "infected" cells and subjected to treatment as indicated above: the cells were seeded in micro-wells in a culture medium containing different concentrations of the samples to be tested with 2.5μCi of 3H-thymidine and 3H-uridine per ml. After 16 h at 37°C, the cells were

25 treated with Trichloroacetic acid, washed in ethanol,

left to dry and counted in 7.5 ml of liquid for scintillation. The antiviral activity of the samples tested is expressed as the minimum concentration required for inhibiting the cytopathogenicity of the virus by 50%: IC50. Furthermore, in order to also evaluate the cytotoxicity of the samples tested, the minimum concentrations necessary for causing morphological damage (observable with an optical microscope) to the cells used, were determined. The comparison was effected vs dextran-sulfate (DS) and the drug Acyclovir (both molecules with a known antiviral effectiveness, therefore used as positive control).

The results obtained are expressed in the table below:

Product tested	Minim. cytotoxic conc. (µg/ml)	Minimum inhibitory concentration IC 50 (µg/ml)						
		Herpes simplex virus-1 (KOS)	Herpes simplex virus-1 (F)	Herpes simplex virus-1 (McIntyre)	Herpes simplex virus-2 (G)	Herpes simplex virus-2 (196)	Herpes simplex virus-2 (Lyons)	Vesicular stomatitis virus
HA-NS1	>400	>400	>400	>400	>400	>400	>400	>400
HAS1	>400	48	16	9,6	9,6	3,2	3,2	1,92
HAS3	>400	16	3,2	1,92	1,92	1,92	1,92	0,128
DS	>400	9,6	9,6	9,6	3,2	3,2	1,92	16
Acyclovir	>400	0,384	0,384	0,64	0,384	0,384	0,384	>80

The experimental data confirm the powerful antiviral action of both HAS1 and HAS3: a comparison between HA-NS1 and HAS1 shows that not all sulfated hyaluronic acids are equivalent as HA-NS1 did not prove to be active, and this
5 difference in efficacy does not depend on the molecular weight or the sulfation degree of hyaluronic acid, it therefore lies in the very structure of HA-NS1 vs HAS1. HAS in fact shows an efficacy equal to that of dextran-sulfate and comparable to that of acyclovir, a reference
10 drug for the treatment of Herpes Simplex. Furthermore, it should be pointed out that acyclovir is inactive vs VSV, whereas HAS degree 1 and 3 has a very powerful antiviral activity vs VSV.

All the samples tested are non-cytotoxic towards the
15 host cell, the minimum cytotoxic concentration obtained is in fact equal to that of the reference drugs normally used in clinical practice for the treatment of Herpes, and proved to be on an average 100 times higher than that proved active in the inhibition of the viral replication.

20 Cytomegalovirus

The activity of the samples tested was determined by evaluating the inhibition of the cytopathogenicity determined by Cytomegalovirus (CMV: AD-169 and Davis
strain) using the previous protocol. The antiviral
25 activity was tested vs HEL cells (pulmonary embryonic

cells) and was expressed as the concentration required for inhibiting the number of plaques formed by the above virus by 50%.

The results obtained are expressed in the table 5 below:

10

Product tested	Minimum inhibitory concentration IC 50 (µg/ml)	
	Cytomegalovirus AD -169 strain	Cytomegalovirus AD Davis strain
HA-NS1	>50	>50
HAS1	5	3,4
HAS3	0,2	0,2
DS	2,7	0,5

15

The table indicates the clear and significant positive result obtained for both HAS1 and HAS3 which again confirms them as effective antiviral agents. Also in this case, HA-NS1 did not prove to be active in inhibiting the proliferation of the virus, confirming the absolute diversity between the two types of degree 1 sulfated product in having an antiviral capacity.

20

Example 14

Evaluation in vitro of the fibrinolytic properties of HAS

25

acid degree 3 with different MW values

The evaluation of the fibrinolytic properties of the products subjected to the test was compared with the acknowledged fibrinolytic activity of the plasmin. In particular, the dissolution rate of the fibrin network and
5 formation of soluble products of the fibrin degradation (FDP), was evaluated.

The plasma samples used come from whole blood of healthy subjects, without any pharmacological treatment underway.

In order to test the fibrinolytic effectiveness of
10 the products subjected to testing, the blood samples were distributed in various test-tubes in which the clot formation was induced.

The formation of FDP was then evaluated after the addition of:

- 15 • plasmin, as control treatment
- HAS3 prepared according to Example 1 and 2
- HAS3 prepared according to Example 4.

Experimental Study

The freshly removed blood samples were distributed
20 in various test-tubes containing sodium citrate in a ratio of 9:1. The test-tubes were immediately centrifuged at 3,000 rpm for 5 minutes. The plasma obtained was transferred to a new test-tube and immediately used for effecting the FDP evaluation.

25 Thrombin (300 mU/ml), preheated to 37°C was added to

the plasma samples, as activator of the fibrinogen in inducing the formation of the clot.

The following were added to the different cuvettes containing the fibrin clot:

- 5 - a plasmin solution 0.5 mU, 5 mU, 50 mU, 500 mU and 1 U
- solutions of HAS3 at concentrations of 25 mg/ml, 50 mg/ml, 100 mg/ml, 150 mg/ml and 200 mg/ml.

The absorbance variation at 405 nm for 60 sec. was evaluated spectrophotometrically for each cuvette. The
10 reaction was left to proceed until the complete dissolution of the clot.

Coagulated plasma, plasmin solution and a solution of HAS3 200KD and HAS3 20KD were mixed in a ratio of 1:1 v/v and maintained at an operating temperature of 37°C

15 **Table 1. Fibrinolytic activity of PLASMIN**

The table indicates the average mAbs/min values registered in the cuvettes containing coagulated plasma to which plasmin has been added at different concentrations. The values specified indicate the rate at which the fi-
20 brin clot is dissolved and consequently the rate at which the FDPs are produced. The fibrinolytic activity of the plasmin is proportional to its concentration.

The average mAbs/min values registered for each concentration of plasmin were subsequently plotted against
25 the respective Units of enzyme to establish the math-

ematic function which correlates the mAbs/min values with the Units of enzyme.

PLASMIN: fibrinolytic activity (mAbs/min)

1 U	500mU	50mU	5mU	0.5mU
190	178	126	68	1

5 Table 2. Fibrinolytic activity of HAS3 at different MW values

The mAbs/min values registered in the cuvettes containing coagulated plasma to which HAS3 had been added at different concentrations, were calculated. The values indicated the rate at which the fibrin clot was dissolved and consequently the rate at which the FDPs were produced. The fibrinolytic activity of HAS3 was proportional to its concentration. In the table, the fibrinolytic activity of HAS3 is expressed as Unit equivalents of Plas-

10

15 min.

HAS3 (200KD) fibrinolytic activity (expressed in mU equivalents of Plasmin)

200 mg/ml	150 mg/ml	100 mg/ml	50 mg/ml	25 mg/ml
1,1 U	50mU	10mU	3mU	0.5mU

HAS3 (10KD) fibrinolytic activity (expressed in mU equivalents of Plasmin)

200 mg/ml	150 mg/ml	100 mg/ml	50 mg/ml	25 mg/ml
400mU	25mU	2mU	0.3mU	0.25mU

5 Results:

The experimentation effected showed how HAS is a powerful fibrinolytic agent, with an effectiveness equal to Plasmin. Plasmin is an important enzyme, belonging to the group of hydrolases, which degrades many proteins of blood plasma and, in particular, the fibrin of the thrombi and of clots. The degradation of the fibrin is called fibrinolysis. A plasmin deficiency can lead to thrombosis, as the thrombi are not adequately degraded. Although HAS is not an enzyme, it has proved to be equivalently active to enzymatic control, thus allowing the use of the sulfated product as a new fibrinolytic agent, having all the advantages of a non-enzymatic molecule such as, for example, stability at room temperature with much longer preservation times and formulation facility.

Example 15

Evaluation of the skin permeation of HAS1 and HAS3 with different MW values.

Evaluation of the skin absorption of diclofenac formulated in association with HAS vs HA

5 The skin used for effecting the experiments is obtained from the abdomen of patients between 30 and 50 years old, subjected to surgical abdominal reductions. The sections of skin with a complete thickness are frozen after the surgical intervention and preserved at -20°C
10 until the moment of the experiment, in which the samples are defrozen at room temperature and accurately separated from the adipose tissue. The skin is divided into square sections of 2.5cm², immersed for a minute in water at a temperature of 60°C and with the help of specific tweez-
15 ers, the corneal layer and epidermis (SCE) are accurately separated from the underlying tissues. The samples obtained are analyzed with an optical microscope and discarded if punctures are found. The SCE is assembled in the lower part of a Franz cell with the epidermis facing
20 downwards and the corneal layer in contact with the donor solution which is situated above. The permeation area is a circular surface of 0.636 cm². The lower and upper part of the Franz cell are accurately fixed to the SCE to separate the donor compartment (volume donor solution:
25 0.50 ml) and acceptor compartment (volume receiver solu-

tion 5.00 ml), whose volumes are calibrated exactly. The solvents to be used are degassed in order to eliminate the formation of bubbles, which must be avoided especially for the receiver solution, which is thermostated at 37°C by means of a circulating thermostatic bath; under these conditions the SCE is at a temperature of 31-33°C. Each experiment is effected triply and the result is intended as the average of the 3, expressed as quantity of analyte which has crossed the surface unit of
10 skin after 24 hours.

The permeation of HA and HAS1 and HAS3 of both MWs was effected from solutions at 3% (weight/volume), with detection of the permeate quantity through the assay of glucuronic acid and ICP (Inductively Coupled Plasma) spectrometry on sulfur, respectively.

The permeation of sodium diclofenac salt was effected on the salt as such in aqueous solution, on the salt in the presence of HA 200 KD at 3% (weight/volume) and on the salt in the presence of HAS3 prepared according to Example 1-2, at 3% (weight/volume). In all three cases, the concentration of sodium diclofenac salt in the permeating solution is equal to 1% (weight/volume). The concentration of diclofenac was determined through reverse-phase HPLC analysis on an apparatus Agilent 1200 Series and UV detection (254 nm), C18 column, eluent ace-

tonitrile/water/acetic acid 50/46/4 with a flow of 1.2 ml/min.

The results obtained are graphed in Figure 10 and Figure 11:

5 Figure 10 shows how the sulfation of HA has significantly increased its permeation through the skin and how this result is particularly evident for the average MW.

Figure 11, on the other hand, shows how HAS behaves as skin absorption promoter of the active principle
10 diclofenac, doubling its total permeate quantity in 24 h with respect to the control and association with HA.

Example 16

Evaluation of the hydrating and protective activity obtained with HAS vs HA and control base cream.

15 20 human subjects aged between 18 and 70 years, with no skin pathologies and pharmacological treatment underway, were treated daily with certain and constant quantities of the products tested, at the level of the forearm.

20 The experimental products were:

- control: consisting of a hydrating base cream
- base cream (as control) containing HA 200 KD 01%
- base cream (as control) containing HAS3 01%, prepared according to Example 1-2

25

Cream Base: composition

	DEIONIZED WATER	85.10 %
DERMOL™ 88	ETHYL HEXYL ETHYLHEXANOATE	6.50
NIKKOMULESE™ 41	POLYGLYCERYL-10 PENTASTEARATE BEHENYL ALCOHOL, SODIUM STEAROYL LACTYLATE	5.00
SEPIGEL™ 305	POLYACRYLAMIDE C13-14 ISOPARAFFIN LAURETH 7	2.50
ISOCIDE™ PF	PHENOXYETHANOL, METHYLPARABEN, ETHYLPARABEN, PROPYLPARABEN, PROPYLENE GLYCOL	0.50
KEMIPURE™ 100	IMIDAZOLIDINYL UREA	0.30
DISODIUM EDTA	DISODIUM EDTA	0.10

Production Method

Charging 90% water and Disodium Edta and Isocide PF.

5 Heating to 65-70°C; in an appropriate container dissolving Nikkomulese, Dermol 88 by heating to 65-70°C; joining the fatty phase to the aqueous phase under the action of a turbine. Cooling to 30-35°C, adding Kemipure 100 dissolved in the remaining 10% of water, adding

10 Sepigel to regulate the viscosity and cooling to 25°C.

The difference in the hydration values obtained was evaluated by means of a corneometer CM825 by an average of 3 points and a profilometric analysis of the skin

surface was also effected using a Visioscan™ VC98 video-camera at time T0 (basal value) and T7, after 7 days of use of the products.

The results obtained are graphed in Figures 12 and 13:

5 Figure 12 shows the greater hydrating effect of the skin after treatment with HAS3, vs both the base cream and vs that containing HA. Figure 13, on the other hand, demonstrates how the skin roughness index, after 7 days of treatment, has significantly diminished with respect to the
10 controls. These data clearly demonstrate the effectiveness of HAS in markedly improving the skin hydration index, showing an efficient hydrating and protective activity on the skin by reducing the transpiration of transepidermal water.

Example 17

15 Preparation of a formulation in the form of a **SOLUTION** for inhalations containing HAS degree 1

40 mg (or 20 mg if the HAS has a MW of 500-730KD) of sulfated hyaluronic acid degree 1, having a low or medium MW, are introduced into a 50 ml glass flask, after which
20 15 ml of PBS 0.2M at sterile pH 7.4, are added. The mixture is subjected to stirring for about 30 minutes, until the complete dissolution of the powder. When the complete dissolution has been obtained, 2 ml of glycol propylene and further PBS 0.2M at sterile pH 7.4 are
25 added until the total volume of 20 ml is reached. The solution is maintained under stirring for a few minutes.

Example 18

Preparation of a formulation in the form of a SOLUTION
for inhalations containing HAS degree 3

100 mg of sulfated hyaluronic acid degree 3 (HAS3)
5 obtained from HA 200 KD are introduced into a 50 ml glass
flask, after which 15 ml of PBS 0.2M at sterile pH 7.4,
are added. The mixture is subjected to stirring for about
30 minutes, until the complete dissolution of the powder.
When the complete dissolution has been obtained, 2 ml of
10 propylene glycol and further PBS 0.2M at sterile pH 7.4
are added until the total volume of 20 ml is reached. The
solution is maintained under stirring for a few minutes.

Example 19

Preparation of a formulation in the form of a HYDROPHILIC
15 GEL containing HAS, HA and CMC

Methyl- and propyl-parabene are dissolved in puri-
fied water at 80°C. After cooling the solution to room
temperature, sodium hyaluronate is added under stirring
until dissolution followed by HAS1 (or HAS3), maintaining
20 the stirring until complete dissolution. Glycerol and
propylene glycol are then added under stirring until com-
plete dissolution. Sodium carboxymethylcellulose (CMC) is
finally added and the mixture is mixed until a gelified
solution is obtained.

25 Example 20

Preparation of a formulation in the form of a **HYDROPHILIC**
GEL for mucosal use (without preservatives) containing
HAS and HA

Sodium hyaluronate is dissolved under stirring, and
5 then HAS1 (or HAS3) in a quantity of water of about 90%
of that envisaged in the formula. Propylene glycol,
Symdiol 68 are added followed by MP Diol Glycol, mixing
until the complete dissolution of the various components.
Carbomer™ 974P is subsequently added and the stirring is
10 maintained until the homogeneous dispersion of the
latter. Beads of sodium hydroxide are dissolved in the
remaining 10% of water and this solution is added slowly
to the that previously obtained, the mixture is mixed to
obtain the gelification of the aqueous phase.

15 **Example 21**

Preparation of a formulation in the form of a **HYDROPHILIC**
GEL containing HAS and Hyaluronidase

Methyl- and propyl-parabene are dissolved in puri-
fied water at 80°C. After cooling the solution to room
20 temperature, the enzyme Hyaluronidase is added under
stirring, followed by HAS3, maintaining the stirring un-
til complete dissolution of the two components. Carbomer
974P is subsequently added and the stirring is maintained
until the homogeneous dispersion of the latter. TEA is
25 then added to obtain the gelification of the aqueous

phase. Glycerol and propylene glycol are finally added under stirring.

Example 22

Preparation of a formulation in the form of a HYDROPHILIC

5 CREAM (Emulsion O/A) containing HAS and Hyaluronidase

The oil phase is prepared by melting liquid paraffin, stearic acid and Tefose 1500 under stirring at 50°C. The aqueous phase is prepared separately by the initial dissolution at 80°C of methyl-parabene and subsequent
10 cooling to room temperature and the final incorporation of glycerol, Hyaluronidase and subsequently HAS3 under stirring until the complete dissolution of the various components.

The aqueous phase is joined to the oil phase and
15 emulsification is effected, the emulsion O/A obtained is cooled under stirring to room temperature.

Example 23

Preparation of a formulation in the form of a FOAM containing HAS and Hyaluronidase

20 Methyl- and propyl-parabene are dissolved in purified water at 80°C. After cooling the solution to room temperature, the enzyme Hyaluronidase is added under stirring, followed by HAS3, maintaining the stirring until complete dissolution. Propylene glycol is then added
25 and the mixture is mixed until complete dissolution;

polyvinylpyrrolidone is subsequently incorporated, mixing until complete dissolution and finally polysorbate 80 maintaining the stirring until dissolution.

The distribution phase of the solution obtained is then effected in a cylinder, proceeding with pressurization with a propellant of isobutane, n-butane, propane.

Example 24

Preparation of a formulation in the form of an OINTMENT containing HAS3 and Hyaluronidase

The base ointment is prepared by melting light liquid paraffin and white Vaseline under stirring at 70°C. After cooling to room temperature, Hyaluronidase is incorporated under stirring, followed by HAS3 and the mixture is mixed until a homogeneous suspension is obtained.

Example 25

Preparation of a formulation in the form of a LIPOGEL containing HAS3 and Hyaluronidase

Light liquid paraffin, white vaseline and cetylstearyl alcohol are melted under stirring at 90°C. The lipogelifying agent hydrogenated castor oil is added under stirring until a homogeneous solution is obtained and the mixture is then slowly cooled to room temperature. Hyaluronidase, HAS3 are finally incorporated and the mixture is mixed until a homogeneous suspension is obtained.

Example 26

Preparation of a formulation in the form of a LIPSTICK
containing HAS and HA

The correct quantity of liquid paraffin indicated in the manufacturing formula is charged into a suitable container. It is heated to 88-92°C and white soft paraffin, hard paraffin, white beeswax, ceresin, arlacel are then added under stirring, the stirring is maintained until the complete melting of the various components. All-rac-a-tocopheryl acetate, allantoin, butylhydroxytoluene, propyl p-hydroxybenzoate are then incorporated and the mixture is mixed until complete dissolution, maintaining the mass at 88-92°C.

The quantity of purified water envisaged in the formula is charged separately into a suitable container, sodium hyaluronate, HAS1 (or HAS3) are then added under stirring until complete dissolution, followed by Disodium Edetate maintaining the stirring until dissolution.

The aqueous phase is transferred under stirring to the container containing the molten mass, maintaining the system at 88-92°C and the stirring until a limpid solution is obtained. The two aromatizing agents are then added under stirring and the mixture is mixed for 10'. The molten mass is poured into moulds and is immediately cooled to $T < 0^{\circ}\text{C}$ until solid sticks are obtained.

Example 27

Preparation of a formulation in the form of VAGINAL
OVULES containing HAS and HA

The gelatine is allowed to swell in 70% of purified water at 85°C; sodium hyaluronate and HAS1 (or HAS3) are
5 dissolved in the remaining quantity of water and this solution is mixed with the glycerine brought to the same temperature. The glycerine solution is added to the swollen gelatine solution and the stirring is maintained until the complete dissolution of the gelatine. The mass is
10 poured into moulds and cooled to $T < 0^{\circ}\text{C}$ until solid ovules are obtained.

Example 28

Preparation of a formulation in the form of a HYDROPHILIC
CREAM (Emulsion O/A) containing HAS and HA

15 The oil phase is prepared by melting liquid paraffin, stearic acid and Tefose 1500 under stirring at 50°C. The aqueous phase is prepared separately by the initial dissolution at 80°C of methyl-parabene and subsequent cooling to room temperature and the incorporation of
20 glycerol, sodium hyaluronate and subsequently HAS1 (or HAS3) under stirring until the complete dissolution of the various components.

The aqueous phase is joined to the oil phase and emulsification is effected, the emulsion O/A obtained is
25 cooled under stirring to room temperature.

Example 29

Preparation of a formulation in the form of an OINTMENT
containing HAS

The base ointment is prepared by melting light liq-
5 uid paraffin and white Vaseline under stirring at 70°C.
After cooling to room temperature, HAS1 (or HAS3) is in-
corporated under stirring, and the mixture is mixed until
a homogeneous suspension is obtained.

Example 30

10 Preparation of a formulation in the form of a HYDROPHILIC
GEL containing HAS3, HA and diclofenac

Methyl- and propyl-parabene are dissolved in puri-
fied water at 80°C. After cooling the solution to room
temperature, sodium diclofenac, sodium hyaluronate and
15 then HAS3 are added under stirring, maintaining the stir-
ring until complete dissolution of the two components.
Carbomer 974P is subsequently added and the stirring is
maintained until the homogeneous dispersion of the lat-
ter. TEA is then added to obtain the gelification of the
20 aqueous phase. Glycerol and propylene glycol are finally
incorporated under stirring.

Example 31

Preparation of a formulation in the form of a HYDROPHILIC
CREAM (Emulsion O/A) containing HAS3 and diclofenac

25 The oil phase is prepared by melting liquid paraf-

fin, stearic acid and Tefose 1500 under stirring at 50°C. The aqueous phase is prepared separately by the initial dissolution at 80°C of methyl-parabene and subsequent cooling to room temperature and the final incorporation
5 of glycerol, sodium diclofenac and subsequently HAS3 under stirring until the complete dissolution of the various components.

The aqueous phase is joined to the oil phase and emulsification is effected, the emulsion O/A obtained is
10 cooled under stirring to room temperature.

Example 32

Preparation of a formulation in the form of a FOAM containing HAS and diclofenac

Methyl- and propyl-parabene are dissolved in purified water at 80°C. After cooling the solution to room
15 temperature, sodium diclofenac is added under stirring, followed by HAS3, maintaining the stirring until complete dissolution. Propylene glycol is then added and the mixture is mixed until complete dissolution; polyvinylpyrrolidone is subsequently incorporated, mixing until complete
20 dissolution and finally polysorbate 80 maintaining the stirring until dissolution.

The distribution phase of the solution obtained is then effected in a cylinder, proceeding with pressurization with a propellant of isobutane, n-butane, propane.
25

Example 33

Preparation of formulations in the form of a skin PATCH
containing HAS and diclofenac

These examples relates to the preparation of a poly-
5 meric matrix containing HAS for the controlled release of
drugs for topic use, and in this case FANS diclofenac so-
dium salt, which improves the dermal and/or transdermal
absorption of the active principle contained therein,
thanks to the promoter action of HAS. The matrix in ques-
10 tion preferably comprises copolymers of acrylic acid and
its acrylic and/or methacrylic esters with a glass tran-
sition temperature (Tg) lower than room temperature,
preferably lower than 0°C, whose free carboxyl groups
present along the polymer chain are salified with organic
15 bases (for example, ammonia, ethylene diamine, copolymers
of acrylic and/or methacrylic acid ester having a cati-
onic ammonium function in the alkyl group (EUDRAGIT® E100
is preferred) or inorganic bases (for example, hydroxides
or carbonate or bicarbonate of alkaline, alkaline-earth
20 and transition metals) as is known to experts in the
field. The copolymers normally used in accordance with
the present invention consist of 2 or more monomers in
variable percentages; examples of these monomers are:

- acrylic acid
- 25 • butyl and/or methyl acrylate

- 2-ethyl hexyl acrylate
- Glycidyl methacrylate
- vinyl acetate

Many copolymers are available on the market (such as
 5 Duro-tak® 280-2416, 280-2516, 87-2620, 87-2852, 380-
 1054, 87-2051, National and Starch) dissolved in organic
 solvents, with a percentage of free carboxyl groups rang-
 ing from 0.1 to 15%, which can be salified with organic
 or inorganic bases, as described above. These copolymers
 10 are preferably contained in the skin patch matrix in an
 amount of 30-90% by weight.

Furthermore, polymeric matrices containing HAS comprising
 the following two main components may be prepared:

-a: Polymethacrylates: i.e. copolymers of acrylic and/or
 15 methacrylic acid ester having a cationic ammonium func-
 tion in the alkyl group (EUDRAGIT® E100, EUDRAGIT® RS and
 EUDRAGIT® RL) are preferred. Said polymers may constitute
 from 10 to 40% by weight of the total of adhesive matrix
 after drying, preferably from 10 to 25%.

20 -b: organic dicarboxylic or tricarboxylic acid (such as,
 for example, succinic, fumaric, adipic and lauric acid),
 as counterion to the cationic component a, (in addition,
 they act as reticulating agents of component a). Compo-
 nent b can be adjusted so that partial or complete neu-
 25 tralization is affected. A further suitable component b

are acid-functional acrylate or methacrylate polymers, for example, polyacrylic acid Carbopol®. Components b may be contained in % in weight ranging from 1 to 40% of the total of the adhesive formulation after drying, preferably
5 from 1 to 20%.

The above polymeric matrix within the final formulation is within the range of 10-90% with respect to the dry weight, preferably from 50 to 90% based on dry weight of the final composition. The quantity of active principle incorporated varies in relation to its nature and the
10 desired dermal or transdermal therapeutic effect. It is normally present in a quantity ranging from 0.1 to 30% by weight with respect to the dry weight of the final composition.

15 The formulation can also contain excipients, lenitives, emollients, emulsifying agents, adhesion modulators, preservatives, plasticizers, acidifiers/buffers. The amounts of the excipients may vary in great ranges, between 0.01 and 30% depending on their function.

20 A skin absorption promoter with lenitive, anti-pruritic and anti-reddening properties is HAS degree 1 or degree 3, preferably degree 3, present in concentrations ranging from 0.1 to 30% of the dry weight of the final composition. The above matrix ensures: a controlled, constant permeation of the active principle without irrita-
25

tion, and adhesiveness to the skin.

Preparation method

1 kg of the methacrylate copolymer selected (for example, Duro-tak® 280-2416, 280-2516 or 87-2852) having a content solid of 30-40% w/w, is added under mechanical stirring with 300g of a 30% w/w EUDRAGIT® E100 water/solvent based solution; the mixture is left under moderate stirring for 30 min. Thereafter, the active principle (100 g of sodium diclofenac salt) and HAS3 previously dissolved in an aqueous solution, are then added. The mixture is left under stirring until the complete dissolution. For the preparation of the matrix layer, this mixture is then laid on top of a film of silicon polyester/paper and drying is effected by evaporation of the residual solvents. The spread matrix has a dry weight of about 60 g/m². The matrix thus obtained is then coupled with a non-woven polyester fabric or polyethylene film for the final formation of the patch.

The final composition of each single patch contains 140 mg of diclofenac and 40 mg of HAS3.

Table

HYDROPHILIC GEL (Example 19)	
Components	Quantity (mg/1g of hydrogel)
HAS1 (HAS3)	40 mg (10 mg)

CMC	20 mg
Glycerol	100 mg
Propylene Glycol	66.75 mg
Sodium Hyaluronate	2 mg
Methyl p-hydroxybenzoate	2 mg
Propyl p-hydroxybenzoate	0.2 mg
Purified Water	up to 1g
HYDROPHILIC GEL for mucosal use (Example 20)	
Components	Quantity (mg/1g of hydrogel)
HAS1 (HAS3)	10 mg
Carbomer 974P	15 mg
Propylene Glycol	100 mg
Sodium Hydroxide	0.33 mg
Sodium Hyaluronate	2 mg
MP-Diol Glycol	37.5 mg
SymDiol™ 68	90 mg
Purified Water	up to 1g

HYDROPHILIC GEL (Example 21)	
Components	Quantity (U.I or mg/1g of hydrogel)
HAS3	10 mg
Hyaluronidase	150 U.I

Carbomer 974P	15 mg
Glycerol	100 mg
Propylene Glycol	66.75 mg
Triethanolamine	13.25 mg
Methyl p-hydroxybenzoate	2 mg
Propyl p-hydroxybenzoate	0.2 mg
Purified Water	up to 1g
HYDROPHILIC CREAM (Emulsion O/A) (Example 22)	
Components	Quantity (U.I or mg/1g of cream)
HAS3	10 mg
Hyaluronidase	150 U.I
Tefose™ 1500	110 mg
Glycerol	80 mg
Stearic Acid	33 mg
Liquid Paraffin	40 mg
Methyl p-hydroxybenzoate	1 mg
Purified Water	up to 1g
FOAM (Example 23)	
Components	Quantity (U.I or mg/1g of solution)
HAS3	10 mg
Hyaluronidase	150 U.I
Polysorbate 80	40 mg
Propylene Glycol	40 mg
Polyvinylpyrrolidone	30 mg

Methyl p-hydroxybenzoate	2mg
Propyl p-Hydroxybenzoate	0.3mg
Purified Water	up to 1g
A cylinder contains about 94% of solution and 6% of propellant (isobutane, n-butane, propane)	
OINTMENT (Example 24)	
Components	Quantity (U.I or mg/1g of ointment)
HAS3	20 mg
Hyaluronidase	200 U.I
Light Liquid Paraffin	200 mg
White Petrolatum	up to 1g
LIPOGEL (Example 25)	
Components	Quantity (U.I or mg/1g of Lipogel)
HAS3	20 mg
Hyaluronidase	200 U.I
Hydrogenated Castor Oil	10 mg
Cetostearyl Alcohol	50 mg
White Petrolatum	365mg
Light Liquid Paraffin	up to 1g

LIPSTICK (Example 26)	
Components	Quantity (mg/1g lipstick)

HAS1 (HAS3)	30 mg (10 mg)
Liquid Paraffin	253.2 mg
White Soft Paraffin	326.2 mg
Hard Paraffin	144.3 mg
Beeswax white	96 mg
Ceresin™	28.2 mg
Arlacel™ 582	95.8 mg
Sodium Hyaluronate	2 mg
Allantoin	1.1 mg
all-rac-α Tocopheryl Acetate	1.1 mg
Propyl p-hydroxybenzoate	0.4 mg
Butylhydroxytoluene	0.4 mg
Purified Water	19.2 mg
Disodium Edetate	1.1 mg
Vanilla Flavour	0.5 mg
Sweet Flavour	0.5 mg
VAGINAL OVULES (Example 27)	
Components	Quantity (mg/1g of ovule)
HAS1 (HAS3)	10 mg
Glycerin	580
Gelatin	200
Sodium Hyaluronate	2
Purified Water	up to 1g
HYDROPHILIC CREAM (Emulsion O/A) (Example 28)	

Components	Quantity mg/1g of cream)
HAS1 (HAS3)	10 mg
Tefose 1500	110 mg
Glycerol	80 mg
Stearic Acid	33 mg
Sodium Hyaluronate	2 mg
Liquid Paraffin	40 mg
Methyl p-hydroxybenzoate	1 mg
Purified Water	up to 1g
OINTMENT (Example 29)	
Components	Quantity (mg/1g of ointment)
HAS1 (HAS3)	20 mg
Light Liquid Paraffin	200 mg
White Petrolatum	up to 1g

HYDROPHILIC GEL (Example 30)	
Components	Quantity (mg/1g of hydrogel)
Diclofenac sodium (*)	30 mg
Carbomer 974P	15 mg
Glycerol	100 mg
HAS3	20 mg

Sodio Ialuronato	2mg
Propylene Glycol	66.75 mg
Triethanolamine	13.25 mg
Methyl p-hydroxybenzoate	2 mg
Propyl p-hydroxybenzoate	0.2 mg
Purified Water	up to 1g
(*): sodium diclofenac 3%	
HYDROPHILIC CREAM (Emulsion O/A) (Example 31)	
Components	Quantity (mg/1g of cream)
Diclofenac sodium	10 mg
Tefose 1500	110 mg
Glycerol	80 mg
HAS3	30 mg
Stearic Acid	33 mg
Liquid Paraffin	40 mg
Methyl p-hydroxybenzoate	1 mg
Purified Water	up to 1g
(*): sodium diclofenac 1%	
FOAM (Example 32)	
Components	Quantity (mg/1g of solution)
Diclofenac sodium	40 mg
Polysorbate 80	40 mg
Propylene Glycol	40 mg

HAS3	10 mg
Polyvinylpyrrolidone	30 mg
Methyl p-hydroxybenzoate	2mg
Propyl p-Hydroxybenzoate	0.3mg
Purified Water	up to 1g
A cylinder contains about 94% of solution and 6% of propellant (isobutane, n-butane, propane)	
(*): sodium diclofenac 4%	

The description being thus described, these methods can obviously be modified in various ways. These modifications should not be considered as diverging from the spirit and perspectives of the invention and all the modifications which can appear evident to a skilled person in the field are included in the scope of the following claims.

CLAIMS

1. Sulphated hyaluronic acid for topical use as a hydrating agent for the treatment therapy of skin pathologies characterized by dryness,
5 lichenification, irritation, pruritus and redness, inflammation and desquamation, said sulphated hyaluronic acid (HA) being obtained by a sulfation involving the alcohol hydroxyls present in the HA polysaccharide chain, starting from a hyaluronic
10 acid (HA) having a molecular weight ranging from 10,000 to 50,000 Da, 150,000 to 250,000 Da or 500,000 to 750,000 Da and a sulphation degree equal to 1 or 3.
2. A pharmaceutical composition for topical use
15 containing sulphated hyaluronic acid as defined in claim 1, together with pharmacologically and/or biologically active agents.
3. The pharmaceutical composition according to claim 2
20 further containing steroids, hormones, proteins, trophic factors, vitamins, non-steroid anti-inflammatory drugs, chemotherapy drugs for topical use, antibiotics, antiviral agents, local anesthetics, anticoagulants, fibrinolytic agents, enzymes and/or other proteases.

4. The pharmaceutical composition according to claim 3 containing hyaluronidase.
5. The pharmaceutical composition according to claim 4 containing polymers.
- 5 6. The pharmaceutical composition according to claim 5 wherein the polymers are hyaluronic acid, carboxymethylcellulose and/or other polymers of a natural or synthetic origin.
- 10 7. The pharmaceutical composition according to any one of claims 2 to 6 in the form of an ointment, lipogel, hydrogel, lipstick, cream, patches, vaginal ovules, vaginal bougies, foam, mucosal gel, ophthalmic preparations, vaginal douches, mouthwash, solutions for topical application or
- 15 inhalation.
8. The pharmaceutical composition according to claim 7 containing sulphated hyaluronic acid as skin absorption promoter of drugs in the form of an ointment, lipogel, hydrogel, lipstick, cream,
- 20 patches, vaginal ovules, vaginal bougies, foam, mucosal gel, ophthalmic preparations, vaginal douches, mouthwash, or solutions.

9. The pharmaceutical composition according to claim 8
in the form of patches containing sulphated
hyaluronic acid in association with non-steroid
anti-inflammatory drugs.
- 5 10. The pharmaceutical composition according to claim 9
containing diclofenac.

Fig. 1

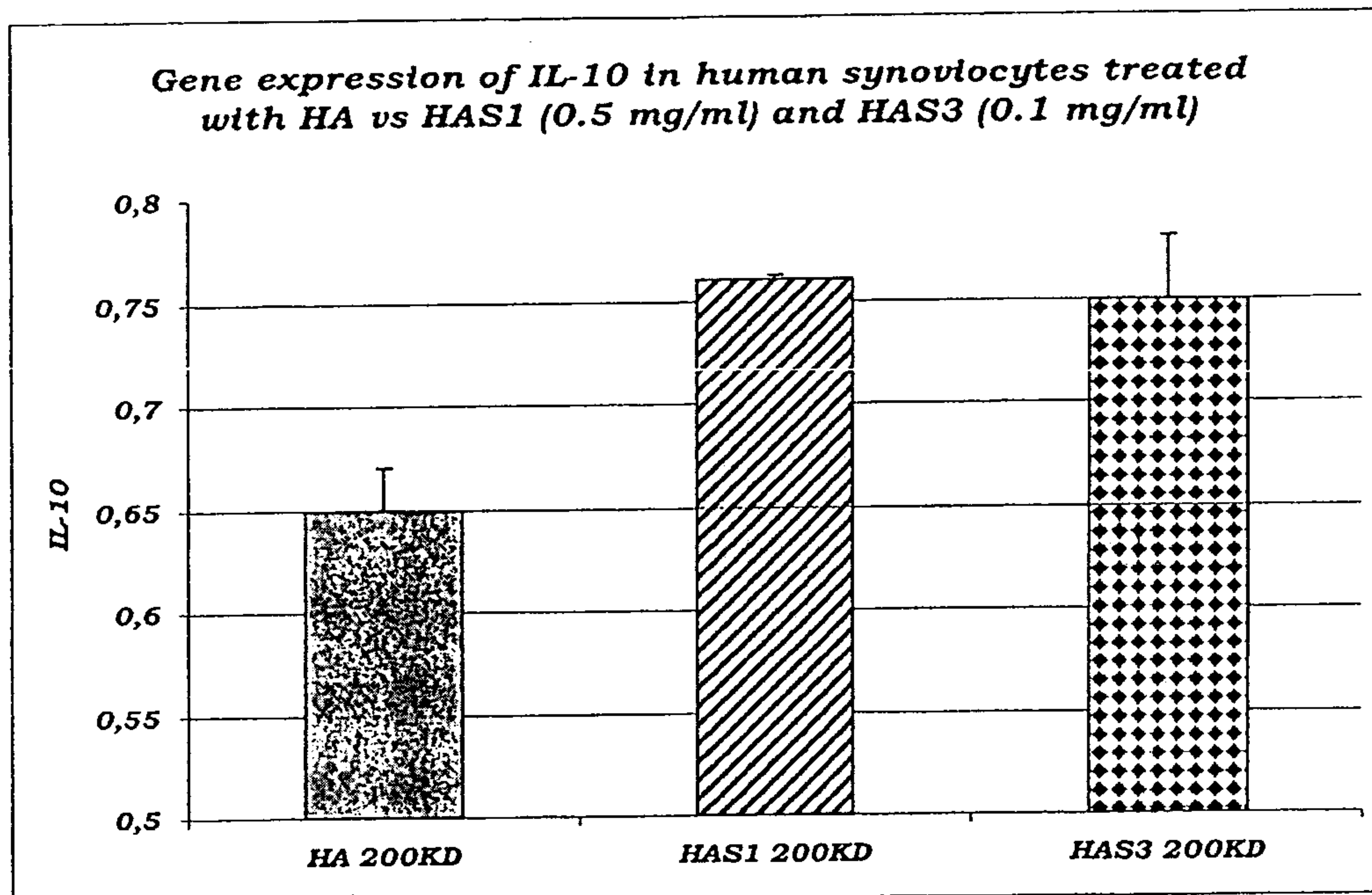


Fig. 2

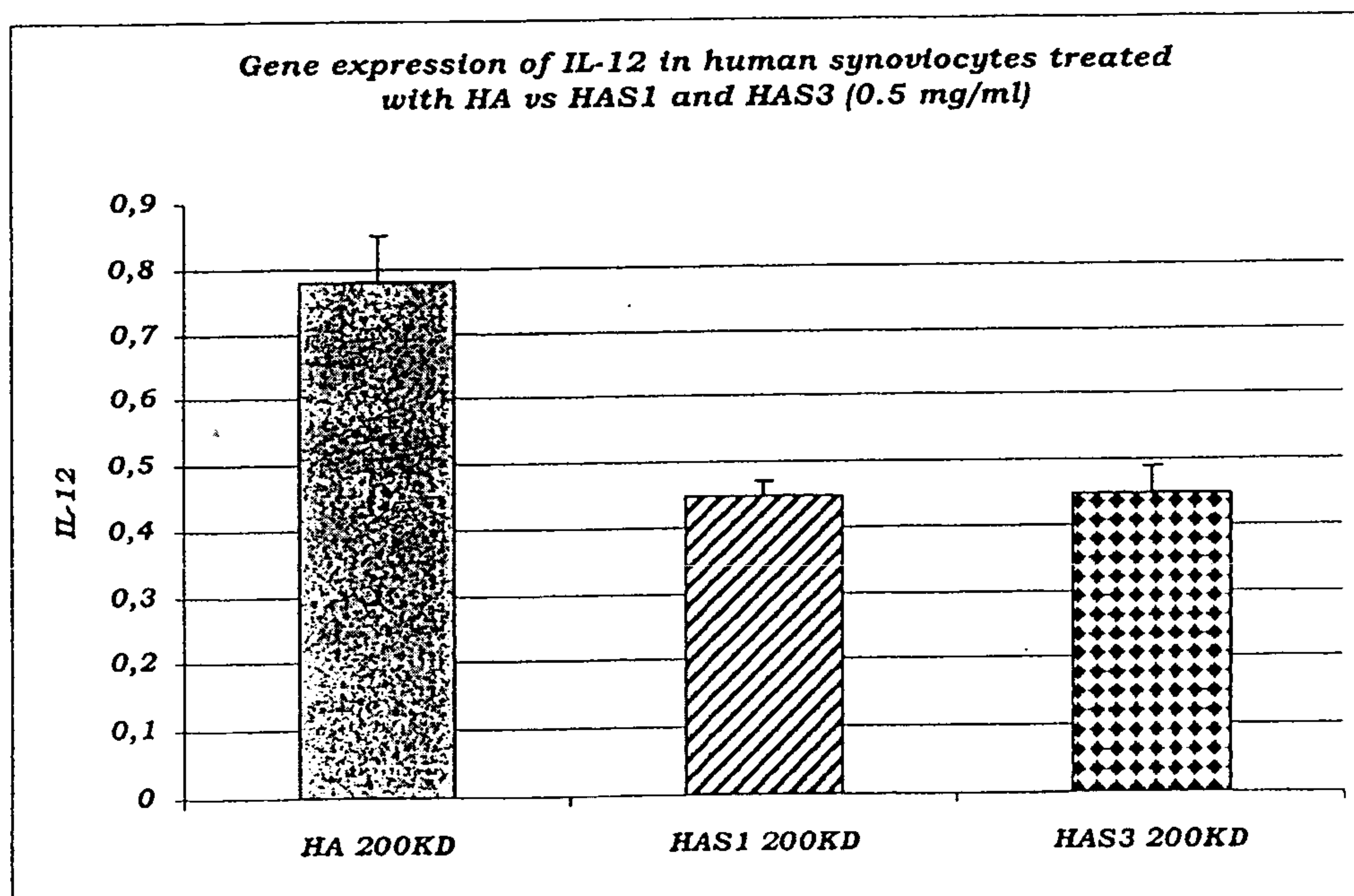


Fig. 3

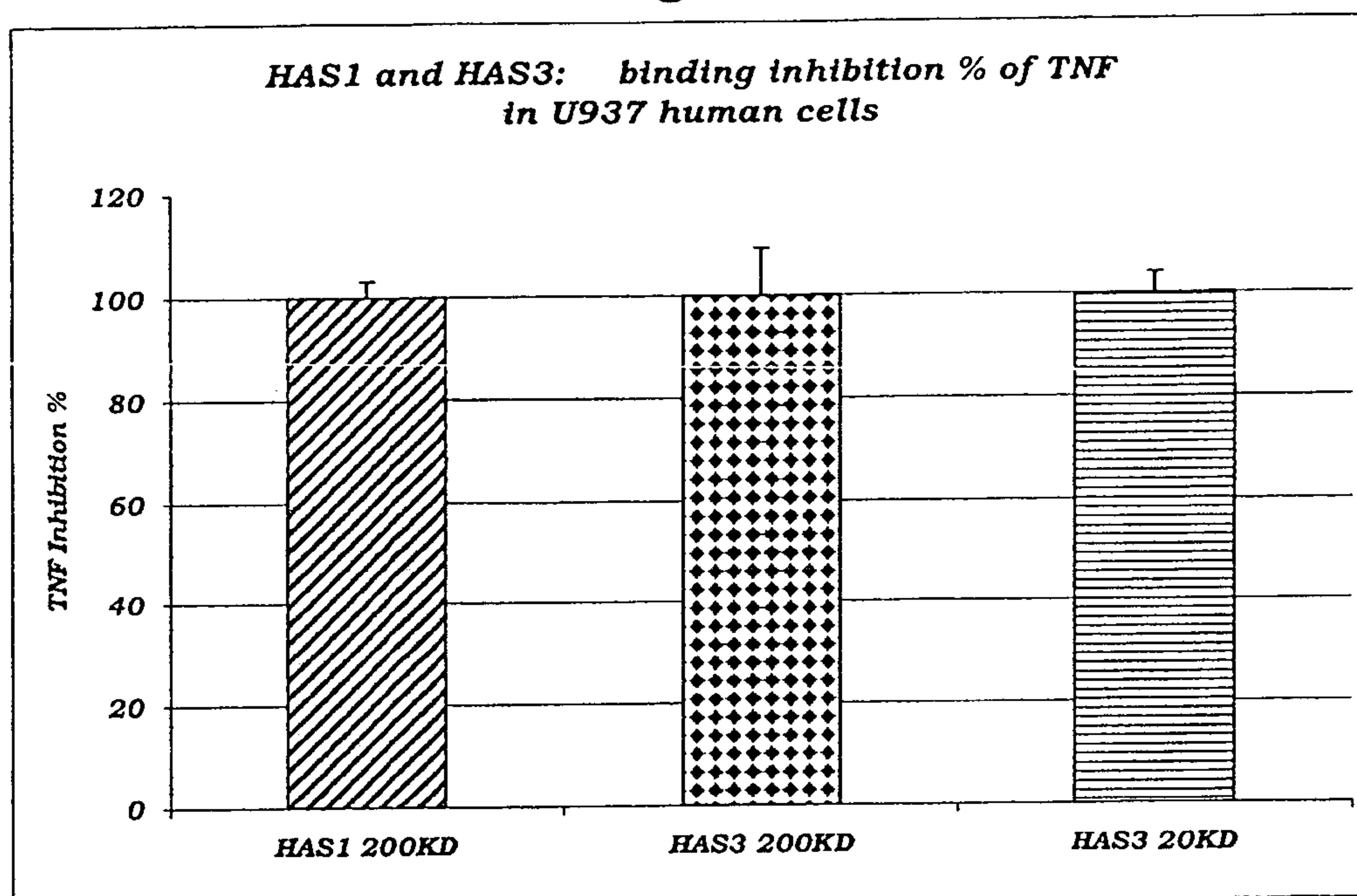


Fig. 4

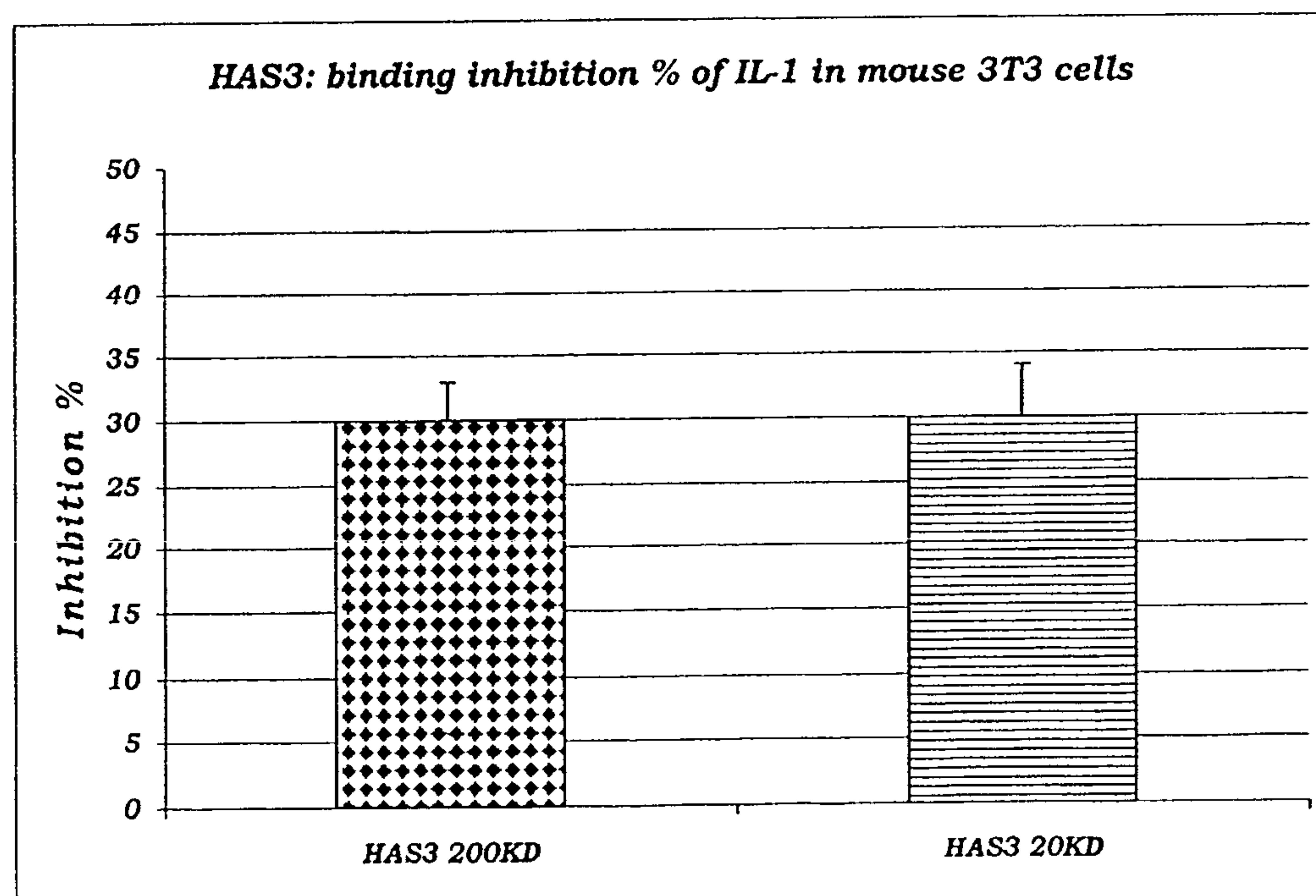


Fig. 5

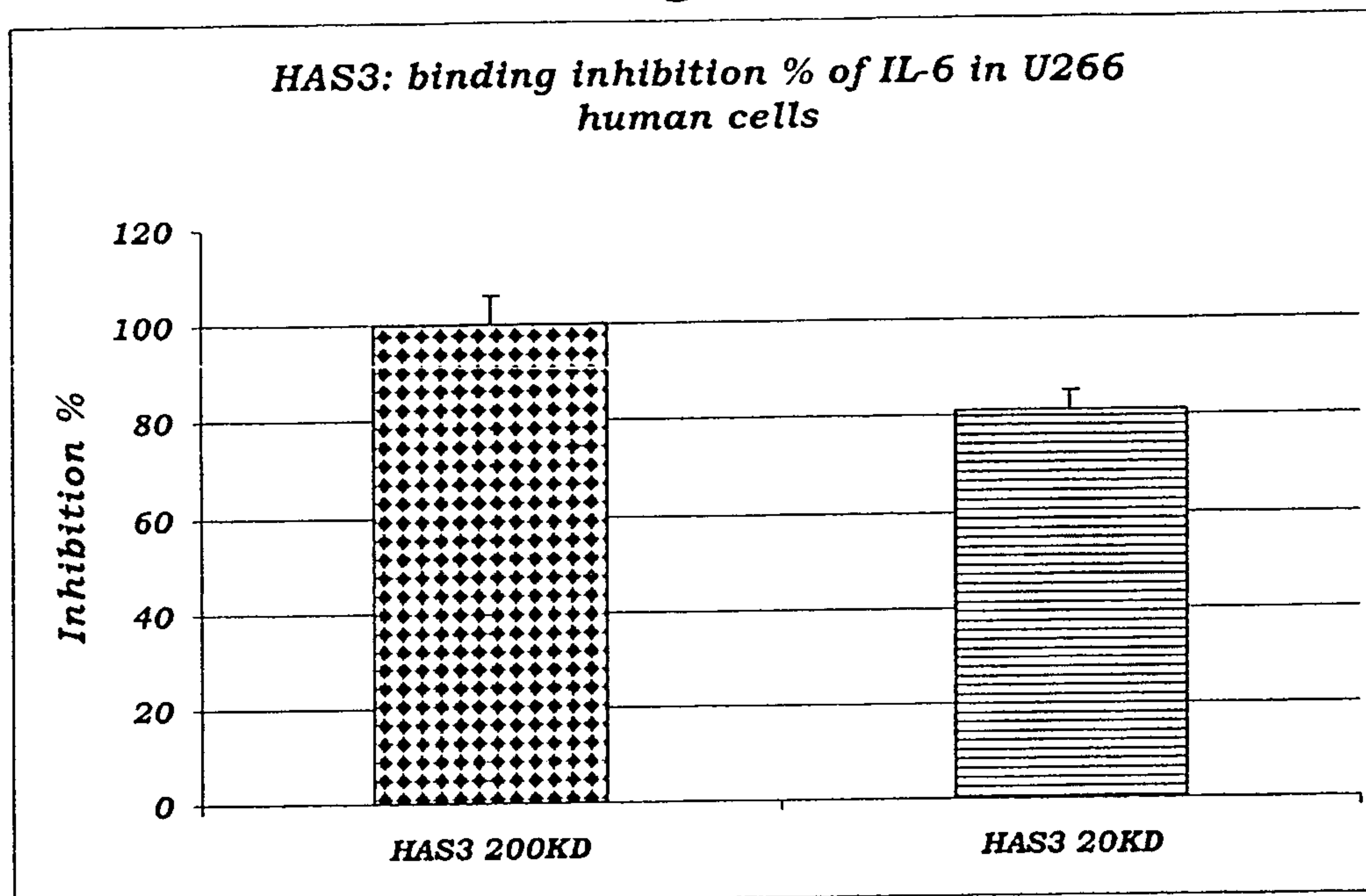


Fig. 6

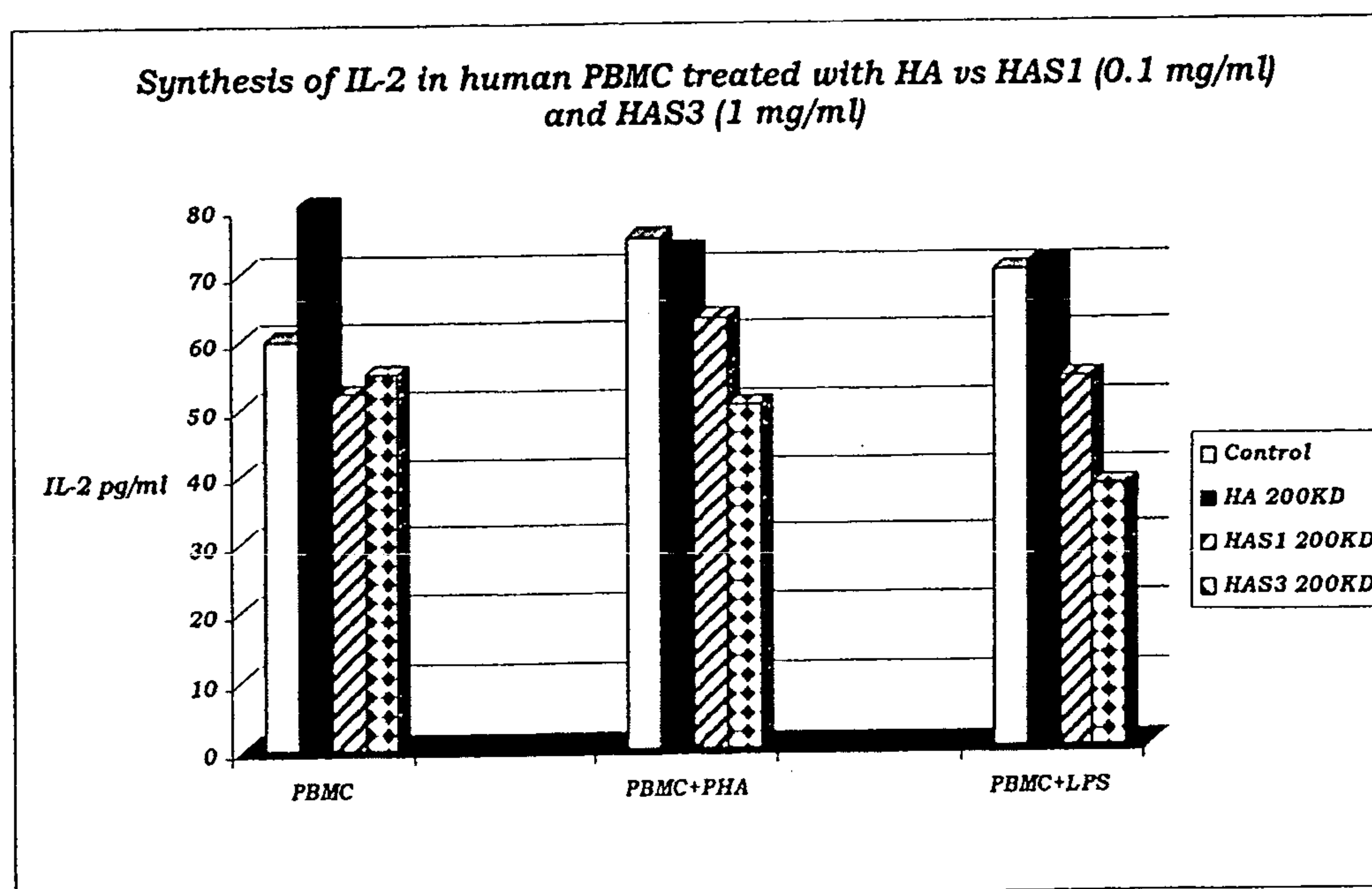


Fig. 7

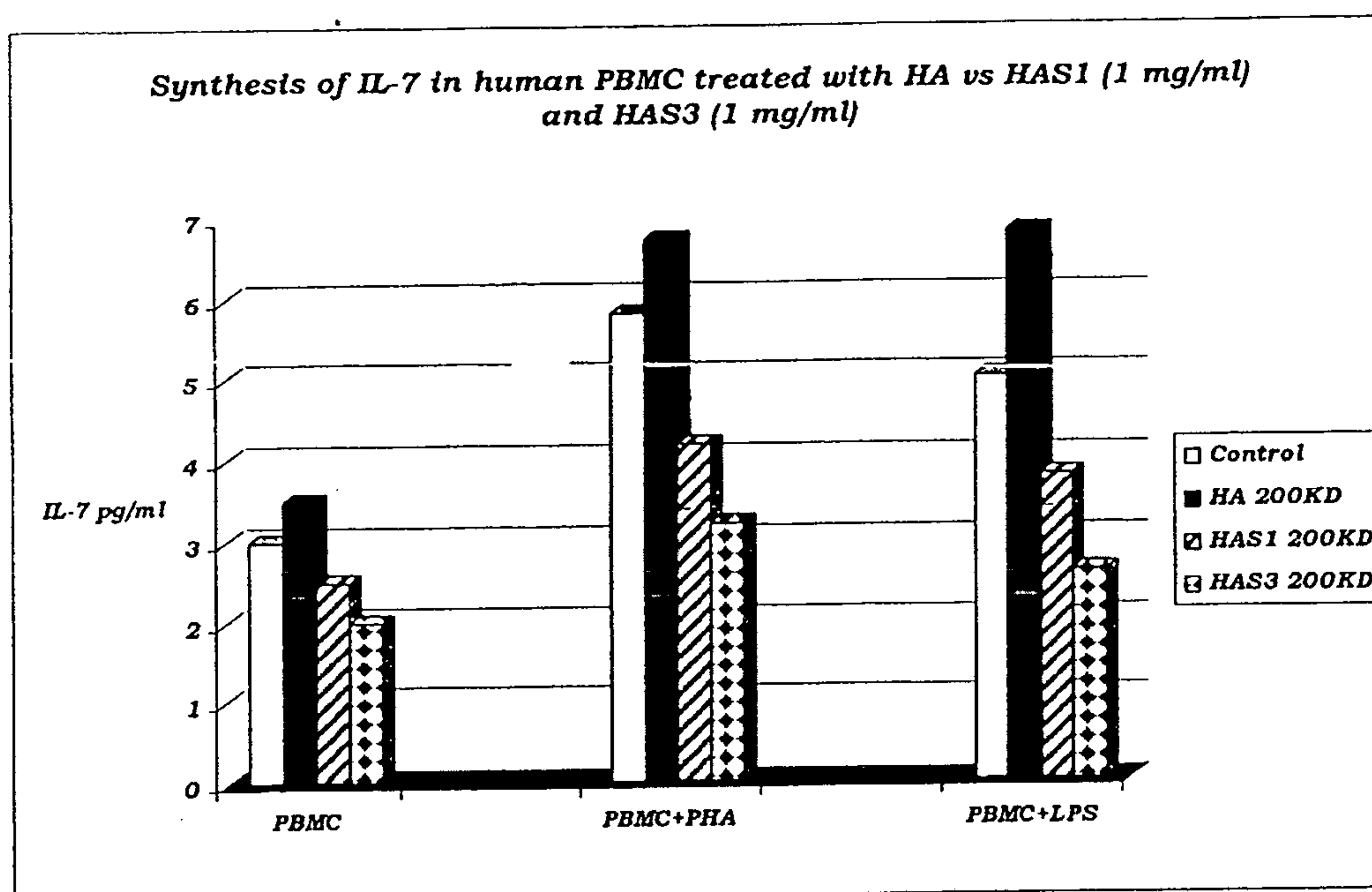


Fig. 8

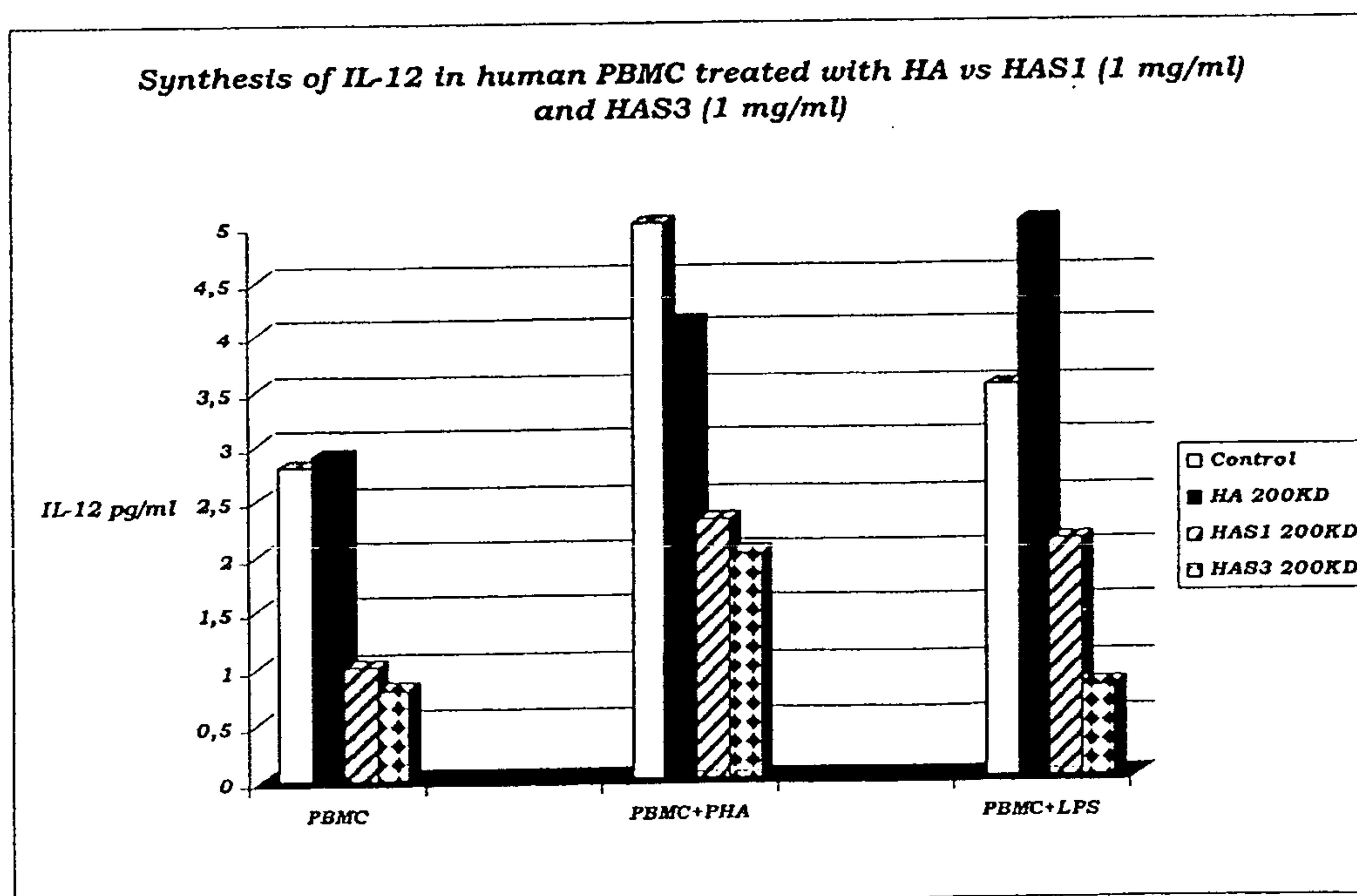


Fig. 9

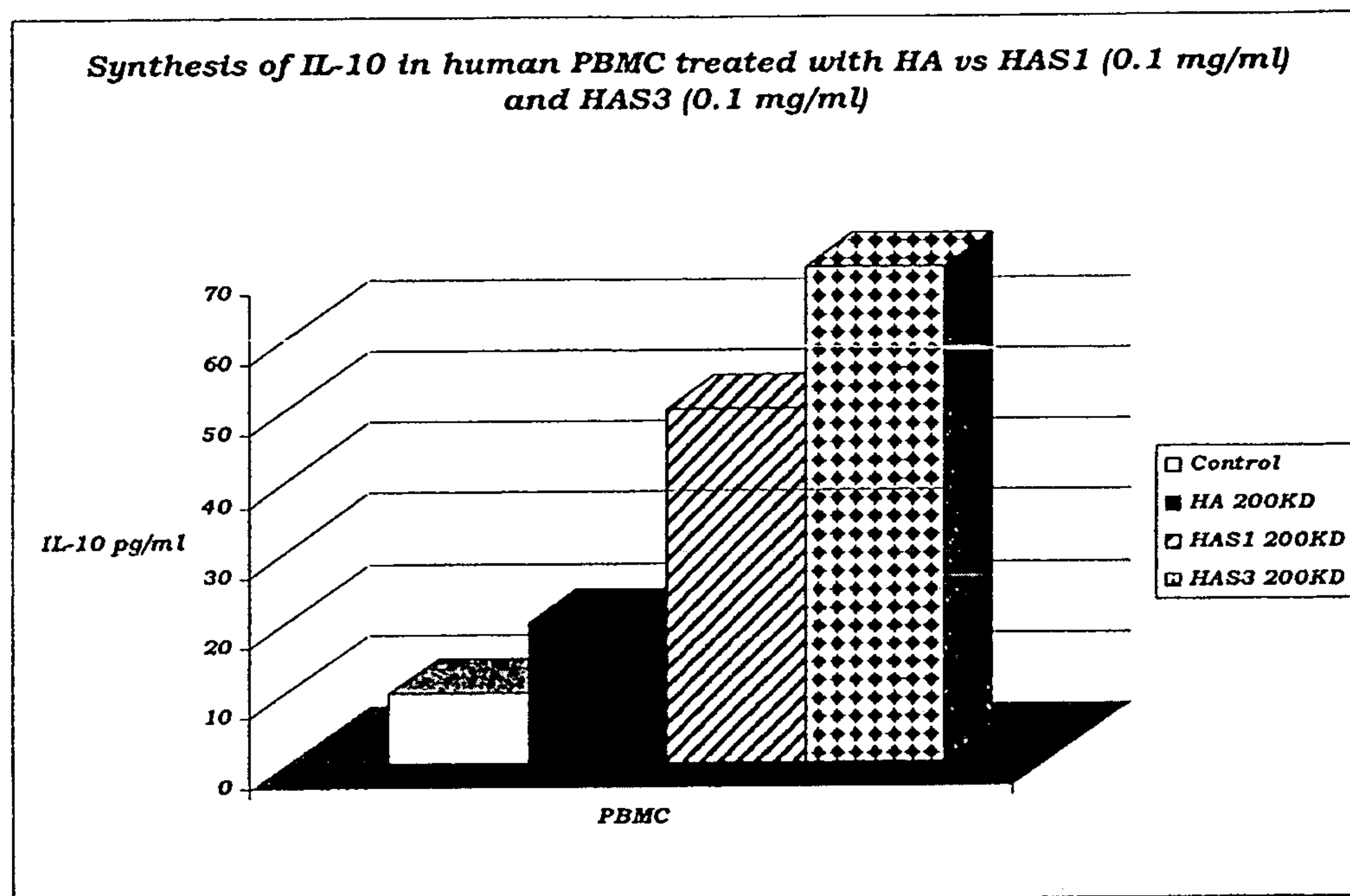


Fig. 10

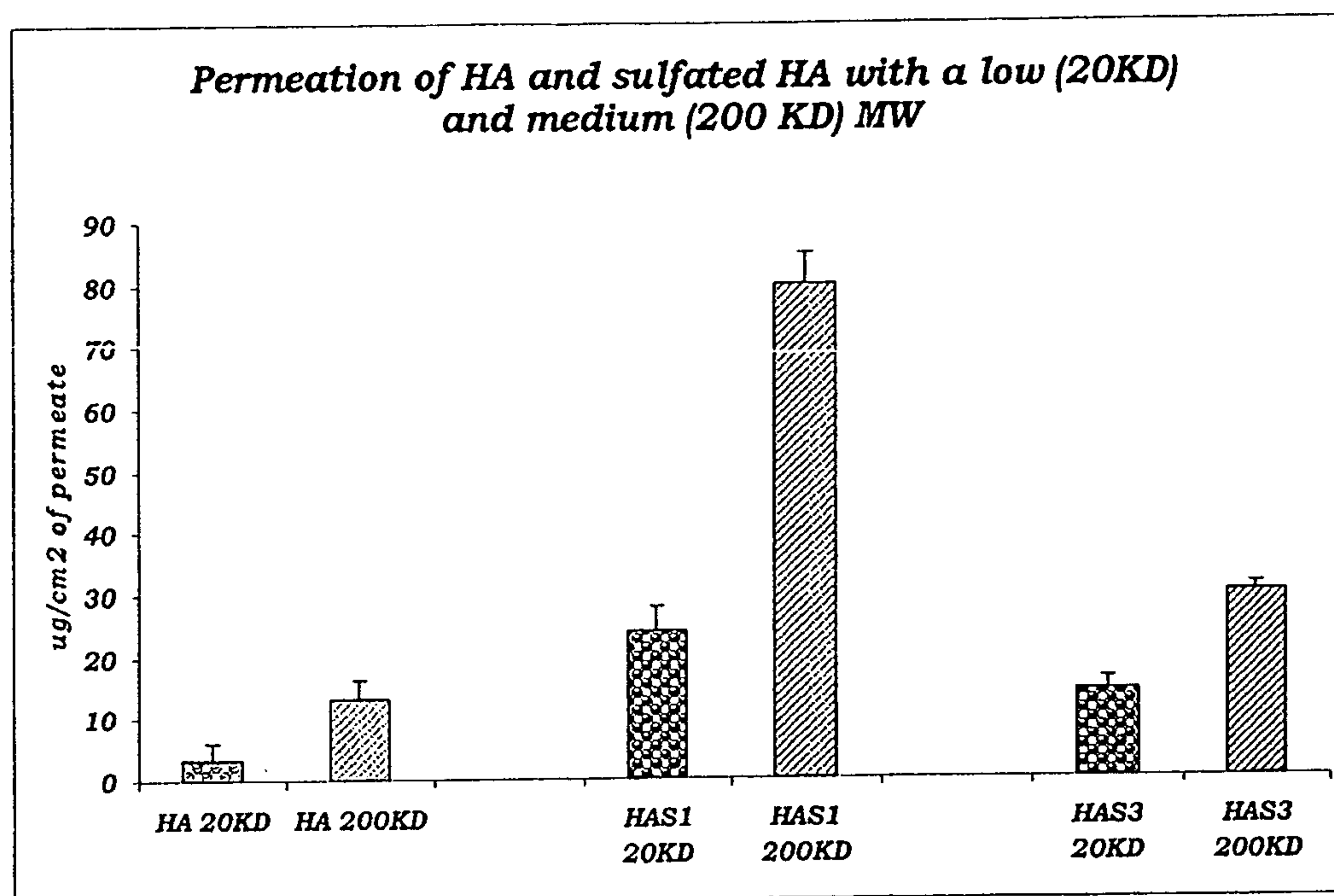


Fig. 11

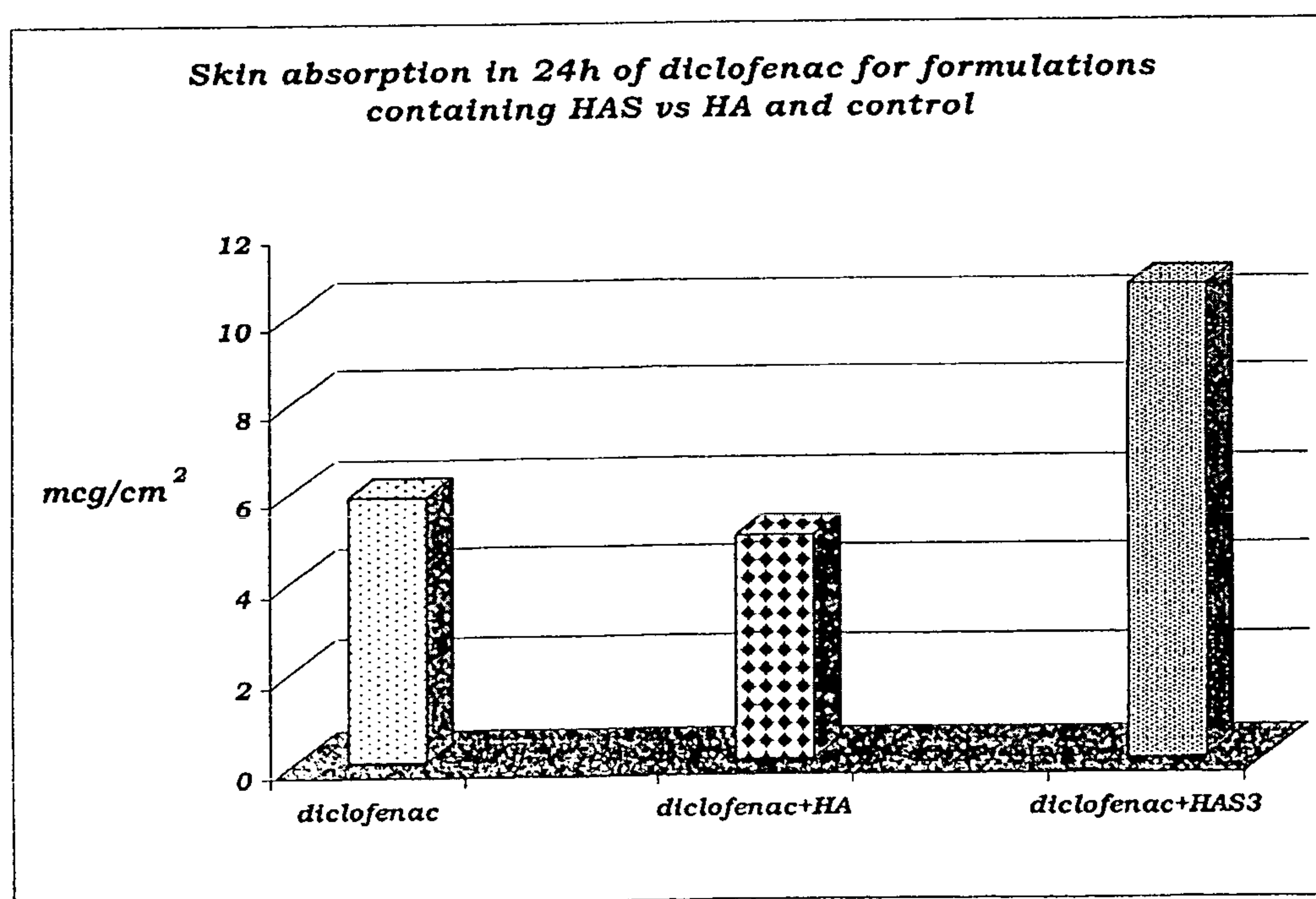


Fig. 12

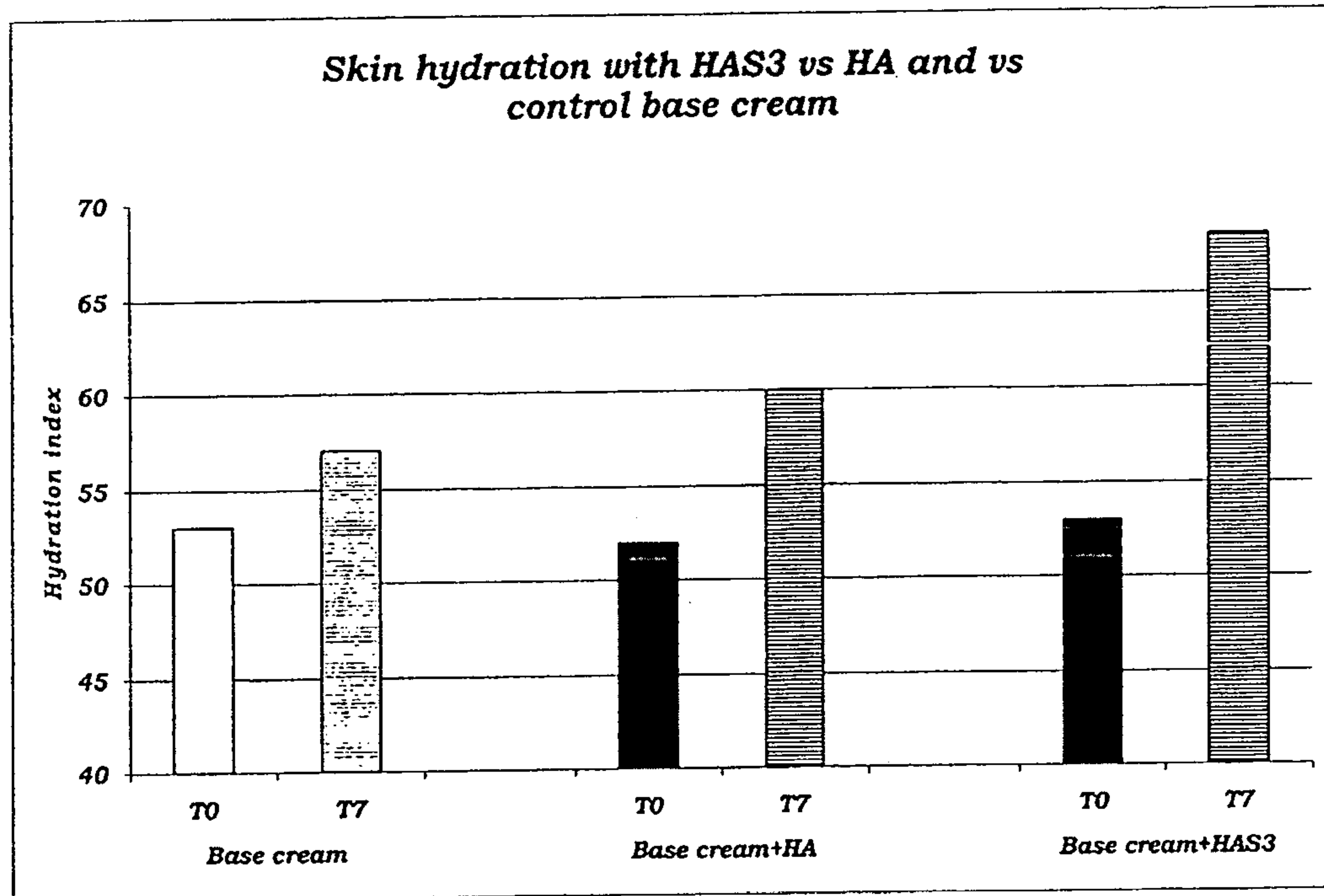


Fig. 13

