(54) Title: A COMPOSITION PROVIDING IMPROVED CLEARANCE OF BIOACTIVE SUBSTANCES FROM THE BLOODSTREAM

(57) Abstract

A pharmaceutical or diagnostic composition comprises, in separate containers, (a) a capturing agent capable of binding to a bioactive substance as well as to a ligand or ligand analogue which is able to bind to a cellular receptor; and (b) a ligand or ligand analogue capable of binding to a cellular receptor as well as to said capturing agent. The composition may be used for providing the rapid clearance of bioactive substances from the circulation.

+ DESIGNATIONS OF "SU"

It is not yet known for which States of the former Soviet Union any designation of the Soviet Union has effect.

1

A COMPOSITION PROVIDING IMPROVED CLEARANCE OF BIOACTIVE
SUBSTANCES FROM THE BLOODSTREAM

5 FIELD OF INVENTION

The present invention relates to a pharmaceutical or diagnostic
composition which, when administered to a subject, is capable
of providing an improved clearance of a bioactive substance
10 from the bloodstream. The invention further relates to methods
of providing such improved clearance for diagnostic or
therapeutic purposes.

BACKGROUND OF THE INVENTION
15

It is known to use antibodies or conjugates of an antibody and
a diagnostically or therapeutically active agent to neutralize
a biologically active substance, such as a toxin (e.g.
lipopolysaccharide; cf., for instance, S.S. Martiny et al.,
20 <u>Crit. Care Med.</u> <u>16</u>, 1988, pp. 629-635), or to diagnose a
specific condition <u>in</u> <u>vivo</u> (cf., for instance, J.F. Chatal,
<u>Monoclonal Antibodies in Immunoscintigraphy</u>, CRC Press,
Florida, USA, 1990), or to target a drug to cells expressing
a specific antigen, e.g. tumour cells (cf., for instance, US
25 4,357,273, EP 336 405 or EP 269 188). Such use of antibodies
for diagnostic purposes suffers from the drawback that the rate
of clearance or elimination of the antibody (conjugate) from
the bloodstream is generally rather slow so that, for an
appreciable period of time, the background activity from the
30 circulating diagnostic agent will be too high to permit an
accurate diagnosis of the condition in question. This, of
course, is a particular disadvantage in case of an acute
condition requiring immediate diagnosis and treatment.
Similarly, when antibodies or antibody conjugates are
35 administered for therapeutic purposes, e.g. to neutralize a
toxin or a drug, it would be a considerable advantage to
eliminate the active substance from the circulation fairly

2

rapidly in cases where the neutralization of the active
substance is less than optimal (this has been described for
lipopolysaccharide, cf. L.S. Young et al., <u>Rev. Infect. Dis.</u>
<u>11</u>, Suppl. 7, 1989, pp. S1564-S1571, or B.J. Appelmelk et al.,
5   <u>Miccrob. Pathogen. 5</u>, 1988, pp. 251-257).

In EP 308 208, it is suggested to use a conjugate of an
antibody which has been modified so that it is able to bind to
a marker produced by a tumour or infectious lesion and a
10   glycoside residue capable of binding to the hepatocyte
asialoglycoprotein receptor for the diagnosis or treatment of
tumours and infectious lesions. The clearance of the conjugate
from the circulation may be deferred by continuously infusing
an inhibitor which, by binding to the same receptor, blocks
15   binding of the conjugate.

The method for providing enhanced clearance suggested in EP 308
208 suffers from the drawback that, even when infusion of the
inhibitor has ceased, it takes some time before it no longer
20   has any effect, i.e. before the hepatocyte receptor is no
longer blocked. This makes the conjugate less favourable for
use in emergency situations where rapid diagnosis and treatment
is essential. Furthermore, it would not be convenient for
patients to whom the conjugate is administered to be subjected
25   to continuous infusion of the inhibitor for a period of time
of up to several days (this is particularly relevant in case
of tumour diagnosis and/or treatment).

The object of the present invention is to provide an improved
30   composition and method for providing a faster rate of clearance
of biologically active substances from the circulation.

SUMMARY OF THE INVENTION

35   Accordingly, the present invention relates to a pharmaceutical
or diagnostic composition comprising, in separate containers,

3

(a) a capturing agent capable of binding to a bioactive substance as well as to a ligand or ligand analogue which is able to bind to a cellular receptor; and

5   (b) a ligand or ligand analogue capable of binding to a cellular receptor as well as to said capturing agent.

In the present context, the term "capturing agent" is used to indicate a substance which, by binding to the bioactive
10   substance, makes it possible to locate, neutralize and/or remove the bioactive substance in question. The term "bioactive substance" is used to indicate any substance which has a biological function or exerts a biological effect in the human or animal body or is formed in the human or animal body in
15   response to a biological process (which might, for instance, be a pathological process) or is released to the circulation in response to a biological process. The term "ligand" is intended to indicate a substance which, in nature, is capable of binding to a particular cellular receptor, while the term
20   "ligand analogue" is used to indicate a substance which is similarly capable of binding to the same cellular receptor. The ligand analogue may be a derivative of the native ligand which has retained its ability to bind to the cellular receptor in question.
25

The term "cellular receptor" is intended to indicate a receptor located on cell surfaces and comprising an extracellular domain with ligand-binding properties. Some receptors have a transmembrane domain which anchors the receptor in the cell
30   membrane and a cytoplasmic domain which generates a cellular signal in response to ligand binding. Other receptors may be anchored to the cell membrane by a phospholipid chain or indirectly by non-covalent binding to transmembrane proteins. Still other receptors are glycolipids which are anchored in the
35   cell membrane by their lipid chains.

In the process of receptor-mediated endocytosis, ligand binding

4

to receptors induces the collection of ligand-receptor complexes in coated pits on the cell surface. These subsequently invaginate to form coated vesicles which fuse with lysosomes followed by enzymatic transformation of the contents of the vesicles (cf. I.H. Pastan and M.C. Willingham, Ann. Rev. Physiol. 43, 1981, pp. 239-250). Endocytotic vesicles may also form from uncoated regions on the plasma membrane (cf. B. Alberts et al., The Molecular Biology of the Cell, Gerland Publ. Inc., New York & London, 1983). Furthermore, phagocytosis may be induced by binding to specific receptors (cf. Alberts et al, op. cit.). Insoluble immunocomplexes may thus be taken up by Kupffer cells via IgG-Fc receptors (cf. B. Smedsrød et al., Biochem. J. 266, 1990, pp. 313-327; S.M. Van der Laan-Klamer et al., Scand. J. Immunol. 23, 1986, pp. 441-447).

The binding between the capturing agent and the ligand or ligand analogue may be effected either by providing the capturing agent with means to bind the ligand or by providing the ligand or ligand analogue with means to bind the capturing agent. Alternatively, the binding between the capturing agent and the ligand or ligand analogue may be effected by providing (complementary) binding means on both the capturing agent and the ligand or ligand analogue.

In another aspect, the present invention relates to a method of providing the rapid clearance of a bioactive substance from the circulation, the method comprising administering, to a subject in need hereof,

(a) an effective amount of a capturing agent capable of binding to a bioactive substance as well as to a ligand or ligand analogue which is able to bind to a cellular receptor,

followed, after a suitable interval, by administration of

(b) an effective amount of a ligand or ligand analogue capable of binding to a cellular receptor as well as to said capturing

agent.

The present method has the advantage that the interval with
which the capturing agent and subsequently the ligand or ligand
5   analogue is administered may be varied at will according to the
time it is estimated to take for the capturing agent to bind
a sufficient amount of the bioactive substance. Likewise, the
method is flexible with respect to the receptor cell through
which the bioactive substance is eliminated, making it possible
10 to avoid overloading a particular organ through which the
bioactive substance would normally be excreted, e.g. the
kidneys. Furthermore, patients receiving this treatment are not
significantly inconvenienced by being subjected to the method.

15 It should be noted that the bioactive substance need not in
each instance be eliminated from the body through receptor
binding and cellular uptake. Rather, the cells may also act as
a storage facility for the bioactive substance resulting in a
protracted release of the bioactive substance to the
20 bloodstream. In this way, the acute toxicity to the excreting
organ (e.g. the kidneys) or normally metabolizing organ (e.g.
the thyroid gland) may be significantly reduced.

The invention further relates to a method of rapid _in vivo_
25 detection of a pathological condition, the method comprising
administering

(a) an effective amount of a capturing agent capable of binding
to a bioactive substance characteristic of the pathological
30 condition in question as well as to a ligand or ligand analogue
which is able to bind to a cellular receptor,

followed, after a suitable interval, by administration of

35 (b) an effective amount of a ligand or ligand analogue capable
of binding to a cellular receptor as well as to said capturing
agent so as to provide a rapid clearance of the capturing agent

from the circulation.

After the capturing agent has been cleared from the
bloodstream, the visualization of any capturing agent binding
5   to the bioactive substance may be carried out by any suitable
means of conducting in vivo diagnosis. The advantage of the
present method is that interference from background activity
caused by circulating capturing agent may be substantially
eliminated within a short period of time, making the method
10 particularly useful for diagnosing conditions where rapid
treatment is essential.

US 4,624,846 discloses a method of localizing a tumour by
injecting a radiolabelled antibody against a tumour marker
15 followed by injection, after a suitable interval permitting
uptake of the antibody by the tumour, of an antibody against
the radiolabelled antibody whereby the level of circulating
radiolabelled antibody decreases by 10-85% in 2-72 hours. This
rate of clearance is rather slow compared to that obtainable
20 with the composition of the present invention, making the
method of US 4,624,846 less suitable for rapid diagnosis.

US 4,634,586 discloses a method of radioimaging leukocytes by
injecting a conjugate of an antibody reactive with a leukocyte
25 surface molecule and a radioisotope chelated with an EDTA or
DTPA derivative followed by the injection of an antibody
against the conjugate in order to clear the conjugate/antibody
complex through the reticuloendothelial system. In a dog study,
a large amount of anti-conjugate antibody injected after
30 injection of the conjugate is reported to result in clearance
of 90% of the conjugate within 15 minutes. It should, however,
be noted that elimination from dog circulation is, at any rate,
faster than elimination from human circulation. Furthermore,
it should be emphasized that the antibody conjugated with the
35 radioisotope must be foreign to the species into which it is
injected as the subsequently injected anti-conjugate antibody
would otherwise not react specifically with the conjugate. This

is not the case with the present composition and method, making
it possible to utilize human (or humanized) substances as the
capturing agent and ligand, thereby significantly reducing the
risk of adverse immune reactions.

In a further aspect, the present invention relates to the use
of a capturing agent capable of binding a bioactive substance
as well as a ligand or ligand analogue which is able to bind
to a cellular receptor for the manufacture, in combination with
a ligand or ligand analogue capable of binding to a cellular
receptor as well as to said capturing agent, of a
pharmaceutical composition providing the rapid clearance of a
bioactive substance from the circulation.

In a still further aspect, the present invention relates to the
use of a capturing agent capable of binding a bioactive
substance characteristic of a pathological condition as well
as a ligand or ligand analogue which is able to bind to a
cellular receptor for the manufacture, in combination with a
ligand or ligand analogue capable of binding to a cellular
receptor as well as to said capturing agent, of a diagnostic
composition providing the rapid clearance of the capturing
agent from the circulation and hence a rapid detection of the
pathological condition in question.

DETAILED DISCLOSURE OF THE INVENTION

In one embodiment of the present invention, it is desired to
clear the capturing agent rapidly from the circulation. This
is particularly the case when the present composition is used
for diagnostic purposes wherein, once the capturing agent is
bound to the bioactive substance, it is a considerable
advantage to eliminate any capturing agent remaining in the
bloodstream quickly in order to avoid excessive background
interference.

In this embodiment, the capturing agent may be monofunctional

8

which is to say that it is able to bind to one substance only.
In order for the diagnostic method of the invention to be
operable, the ligand or ligand analogue may have to be modified
so as to be able to bind to the capturing agent as well as to

5   the receptor. Alternatively, the bioactive substance and the
ligand may be substantially identical. In the latter case,
administration of the bioactive substance, which when it
circulates will act as a ligand binding to a specific cellular
receptor, subsequently to the administration of the capturing

10 agent will result in the clearance of unbound capturing agent
from the circulation. Apart from this, the capturing agent may
be bifunctional which is understood to mean that it is capable
of binding to two different substances (typically the bioactive
substance and the ligand or ligand analogue which will be

15 different). Also when the capturing agent is bifunctional, the
ligand may be modified so as to be able to bind to the
capturing agent.

In another embodiment of the present invention, it is desired
20 to clear the bioactive substance rapidly from the circulation.
This is particularly the case when the present composition is
used for therapeutic purposes where binding to the capturing
agent does not neutralize all the biological activity of the
bioactive substance or where the binding affinity of the

25 capturing agent is not high enough to reduce the free
concentration of the bioactive substance to any significant
extent. In such a case clearance of the bound bioactive
substance from the bloodstream will be important.

30 In this embodiment, the capturing agent may be monofunctional
(as defined above), in which case the ligand or ligand analogue
will have to be modified so that it is able to bind both the
capturing agent and the specific cellular receptor. The
bioactive substance will be different from the ligand or ligand

35 analogue. Alternatively, the capturing agent may be
bifunctional (as defined above), in which case the the
bioactive substance and the ligand or ligand analogue will

typically be different. Also when the capturing agent is bifunctional, the ligand may be modified so as to be able to bind to the capturing agent.

5   In case the capturing agent is monofunctional, it may comprise an antibody which is reactive with the bioactive substance, or a cellular receptor (other than the specific ligand-binding receptor) or a fragment thereof including the binding site for the bioactive substance. The ligand or ligand analogue may then
10 be modified so as to comprise an antibody (including an anti-idiotype antibody), a cellular receptor (other than the specific ligand-binding receptor) or a fragment thereof including the binding site for the capturing agent, or wherein the ligand or ligand analogue is modified with a bioactive
15 substance or a derivative or fragment thereof capable of binding the capturing agent.

In the present context, the term "antibody" is used to indicate any substance formed in the human or animal body or by a human
20 or animal cell as a response to exposure to an antigen. The antibody may be a polyclocal or monoclonal antibody or may be one prepared by recombinant DNA techniques. The term also includes antibody fragments, such as Fab', $F(ab')_2$ or Fv fragments as well as single-domain antibodies.
25

Monoclonal antibodies may be obtained by well-established methods, e.g. as described in A. Johnstone and R. Thorpe, Immunochemistry in Practice, 2nd. Ed., Blackwell Scientific Publications, 1987, pp. 35-43. When prepared by recombinant DNA
30 techniques, the antibody may be produced by cloning a DNA sequence coding for the antibody or a fragment thereof into a suitable cell, e.g. a microbial, plant, animal or human cell, and culturing the cell under conditions conducive to the production of the antibody or fragment in question and
35 recovering the antibody or fragment thereof from the culture. Possible strategies for the preparation of cloned antibodies are discussed in, for instance, L. Riechmann et al., Nature

332, 24 March 1988, p. 323 ff., describing the preparation of
chimeric antibodies of rat variable regions and human constant
regions; M. Better et al., Science 240, 20 May 1988, p. 1041
ff., describing the preparation of chimeric mouse-human Fab
fragments; A. Sharra and A. Plückthun, Science 240, 20 May
1988, pp. 1038-1040, describing the cloning of an
immunoglobulin Fv fragment containing antigen-binding variable
domains; and E.S. Ward et al., Nature 341, 12 October 1989, pp.
544-546, describing the cloning of isolated antigen-binding
variable domains ("single-domain antibodies").

The term "anti-idiotype antibody" refers to an antibody which
is reactive with the epitope binding site of another antibody.
The anti-idiotype antibody may be prepared by any one of the
methods indicated above.

In certain cases where the ligand or ligand analogue is
modified with a bioactive substance, it may be necessary to
modify the bioactive substance in order to reduce the risk of
adverse effects arising from the administration of the modified
ligand. Thus, if the bioactive substance is one which, in the
unmodified state, would be toxic to the human or animal body,
one may suitably employ a detoxified derivative thereof, e.g.
a detoxified fragment thereof. It may, in any case, be
preferred to use fragments of the bioactive substance as this
will reduce the size of the modified ligand molecule.

When the capturing agent in the composition of the invention
is bifunctional, it may suitably comprise a bispecific
antibody, i.e. an antibody which is reactive with two different
antigens or epitopes. The bispecific antibody may, for
instance, be synthesized chemically by cross-linking antibodies
of different specificities or by combining one antibody
fragment containing at least one epitope-binding site
responsible for the reactivity of the antibody with the
bioactive substance with another antibody fragment containing
at least one epitope-binding site responsible for the

reactivity of the antibody with the ligand or ligand analogue, to form a hybrid antibody (cf., e.g., EP 179 872). Alternatively, bispecific antibodies may be formed by fusing two or more hybridomas, e.g. polydomas (cf., for instance, NL 86-1542, EP 68 763 or EP 338 497), or by recombinant DNA techniques (e.g. as described by W.D. Huse et al., Science 246, 1989, pp. 1275-1281 or E.S. Ward et al., Nature 341, 1989, pp. 544-546).

10 The bifunctional capturing agent may also comprise

a conjugate of an antibody and a cellular receptor (other than the specific ligand-binding receptor) or a fragment thereof comprising the binding site for the bioactive substance, a single-stranded oligonucleotide, an intercellular adhesion molecule, or a complexing agent (e.g. biotin, avidin, lectin or a drug), or

a conjugate of a cellular receptor (other than the specific ligand-binding receptor) or a fragment thereof comprising the binding site for the bioactive substance and a single-stranded oligonucleotide, an intercellular adhesion molecule, or a complexing agent (e.g. biotin, avidin, lectin or a drug).

25 In this case, too, the ligand or ligand analogue may be modified so as to comprise an antibody, a cellular receptor (other than the ligand-binding receptor) or a fragment thereof comprising a binding site for the capturing agent, a single-stranded oligonucleotide, an intercellular adhesion molecule or a complexing agent, each of which is capable of forming a conjugate with the part of the capturing agent which does not bind the bioactive substance.

Thus, when the capturing agent comprises a single-stranded oligonucleotide, the ligand or ligand analogue will typically be modified with a complementary single-stranded oligonucleotide. Likewise, when the capturing agent comprises

12

an intercellular adhesion molecule or a complexing agent, the
ligand or ligand analogue will usually be modified with a
substance capable of binding the intercellular adhesion
molecule and complexing agent (e.g. avidin or streptavidin if

5   the capturing agent is biotin, biotin if the capturing agent
is avidin, a carbohydrate if the capturing agent is a lectin,
etc.), respectively. Suitable lectins may be derived from plant
(cf. H. Lis and N. Sharon, Ann. Rev. Biochem. 42, 1973, pp.
541-551) or mammalian sources (cf. B. Smedsrød et al., Biochem.

10   J. 266, 1990, pp. 313-327, or Magnusson et al., Biochem. J.
257, 1989, pp. 651-656).

For diagnostic purposes, the capturing agent may suitably be
provided with a label. The substance used to label the

15   capturing agent may for instance be a radioactive isotope such
as In-111, Tc-99m, I-131, I-125 or I-123 (cf. US 4,357,273, EP
336 405 or EP EP 269 188). Alternatively, the capturing agent
may be labelled with a substance useful for magnetic resonance
imaging. Examples of such substances are magnetite particles

20   which may be coated with the antibody and used for imaging
substantially as described in S. Cerdan et al., Magnetic
Resonance in Medicine 12, 1989, pp. 151-163, or paramagnetic
atoms, e.g. C^{13} or gadolinium (cf., for instance, J.C.
Saccavini et al., Invest. Radiol., Suppl. 1, 1989, pp. S292-

25   S293, or P. Shrere and A. M. Aisen, Magnetic Resonance in
Medicine 3, 1986, pp. 336-340).

The cellular receptor to which the ligand or ligand analogue
binds after binding to the capturing agent may be any receptor

30   found in the body although it may advantageously be a receptor
which is found in an organ through which waste products of the
body are usually eliminated, e.g. the liver, the spleen, the
bone marrow, the lungs, the kidneys, the skin or the placenta.

35   The liver has an important function as a scavenger in the body
(cf. B. Smedsrød et al., op. cit.). In the liver, the
population of endothelial cells and Kupffer cells compose the

13

hepatic reticuloendothelial system which is responsible for the uptake of various macromolecules, including nanoparticles and particulate matter, respectively (cf. E. Wisse et al. (Eds.), Cells of the Hepatic Sinusoid, Vol 2, The Kupffer cell
5 Foundation, Rijswijk, The Netherlands, 1989). Receptors responsible for the binding of such molecules include, i.a., the hyaluronan, collagen, PIIINP (N-terminal propeptide of collagen type III), mannose/N-acetylglucosamine, complement, asialoglycoprotein and IgG Fc receptors (cf. B. Smedsrød et
10 al., op. cit.). Binding of ligands to these receptors results in receptor-mediated endocytosis whereby vesicles containing the ligand-receptor complex are fused with lysosomes and subsequently degraded. Transcytosis to the parenchymal cells is another possibility (cf. B. Smedsrød et al., op. cit.) as
15 is direct phagocytosis of the Kupffer cells which sequester particulate matter. A further suitable receptor is the hepatic receptor for tissue plasminogen activator.

Identical or similar reticuloendothelial cells may also exist
20 in other organs such as the spleen, the lungs, the bone marrow or the placenta. The cells in these organs may be equipped with special receptors characteristic of the organs.

Other types of receptors include receptors for peptide or
25 polypeptide hormones and growth factors shown in Table I below.

Table I

30 Receptors for growth factors	epidermal growth factor, transforming growth factor, insulin-like growth factor, platelet-derived growth factor, interleukin-1α, interleukin-1β, interleukin-2, colony stimulating factor, γ-interferon, nerve growth factors, tumour necrosis factor

14

Receptors for peptide hormones	T3, T4, oxytocin, histamine, vasopressin
Receptors for polypeptide hormones	growth hormone, insulin, glucagon, thyroid-stimulating hormone, follicle-stimulating hormone, luteinising hormone, adrenocorticotropic hormone, parathyroidea hormone, prolactin, lipotropin, cholecystokinin, calcitonin, secretin, atrial natriuretic factor, endothelin, vasoactive intestinal polypeptide, transferrin, tachykinin
Intercellular adhesion factors	intercellular adhesion molecule 1, endothelial leukocyte adhesion molecule 1, vascular cell adhesion molecule 1

When the corresponding ligands bind to these receptors, an intracellular signal is triggered in response. When such receptors are used for the elimination of the bioactive substance, care should be taken that the binding of the conjugate of the bioactive substance, capturing agent and ligand to the receptor should not trigger an intracellular signal to such a degree that it has any significant overall physiological impact.

Another group of receptors are those located intracellularly which mainly bind hydrophobic ligands. Examples of such receptors are shown in Table II below

Table II

Receptors for vitamins	Vitamin A, Vitamin D, retinoic acid
Receptors for fatty acid derivatives	prostaglandin B1, D2, E1, E2, F2, leukotriene, thromboxane B2
Receptors for steroid hormones	t e s t o s t e r o n e , progesterone, cortisol, estradiol, aldosterone

A third group of receptors are those which are located on cells of the nervous system, e.g. α-adrenergic, β-adrenergic, epinephrine, adrenalin, dopamin, opiate (such as morphine, enkephaline, endorphin) angiotensin, adenosin, benzodiazepine, bradykinin, choline muscarinic, cholinergic nicotinic, GABA or serotonin receptors. These receptors may, in certain cases, be used for the elimination of the bioactive substance provided that they are sufficiently abundant and not protected by the blood-brain barrier. In this case, too, it is important that the physiological receptor response does not have any significant overall impact on the body.

The spleen forms part of the reticuloendothelial clearance syrtem and as such includes similar cells as those described above for the liver sinusoids. However, the spleen may also contain specific receptors which are different from those of the liver. Apart from this general clearance function, the spleen sequestrates aged erythrocytes. The signal for the retention of erythrocytes in the spleen is not known (M.R. Clark, Physiol. Rev. 68, 1988, pp. 503-554), but the presence of a senescent antigen derived from the band 3 protein in combination with auto-antibodies against this antigen may be the signal that triggers the sequestration, e.g. via the Fc receptors (M.M.B. Kay, Proc. Natl. Acad. Sci. USA 81, 1984, pp. 5753-5757).

16

Apart from these receptors, other receptors are found in the spleen, such as corticotropin-releasing factor receptors, Fc receptors, atrial natriuretic peptide receptors, the interferon receptor, substance P receptors and tachykinin receptors.

Receptors found in the kidneys include angiotensin, vasopressin, neuropeptide Y, bradykinin, vasoactive intestinal peptide, endothelin, prostaglandin E2, mineralocorticoid, α-adrenergic, dopamine-1, serotonin, β-1 integrin or thiazide diuretic receptors. The mechanism for selective excretion includes binding of the conjugate of the bioactive substance, capturing agent and ligand to a specific kidney receptor, intracellular degradation or modification followed by release to the tubular cells with subsequent secretion.

The lungs have the largest blood flow of all organs and would therefore be well suited for excretion of evaporatable substances. Reticuloendothelial cells are also present in the lungs. Examples of lung receptors which may be suitable for clearance of bioactive susbtances in this fashion are histamine, endothelin, β-adrenergic, interleukin-1, interferon-γ, lung vasoactive intestinal peptide, muscarinic, adenosine, substance P, benzodiazepine, atriopeptin III or leukotriene receptors.

Examples of bone marrow receptors which might be useful for the present purpose are the Fc gamma RI, RII and RIII, interleukin 1 and 2, erythropoietin, transferrin, leukotriene, benzodiazepine or somatostatin receptors.

Due to the extremely large surface area of the skin, it may be a particularly appropriate target for depositing toxic components which cannot be excreted through any other organ without causing damage. Deposited toxic components may either be gradually re-routed to the bloodstream and excreted through another organ (typically the liver) in doses which are small enough not to cause any damage to that organ. The sweat glands

may represent a direct route of excretion via appropriate receptors located in the glands (e.g. a vasoactive intestinal polypeptide-like receptor, cf. W. Kummer et al., Neurosci. Lett. 110, 1990, pp. 239-243). Examples of receptors on keratinocytes and skin fibroblasts which might be suitable for the present purpose are low-density lipoprotein, transferrin, interleukin-1 and -2, histamin H1 and H2, glycocorticoid, vitamin D, retinoic acid or retinoic acid gamma receptors or integrin receptors for fibronectin, collagen or laminin.

Intrauterine treatment of foetal disorders is believed to be feasible by the clearance method of the invention by utilizing one of the large number of receptors in the placenta. Examples of such receptors are Fc-γ, LDL, hemopexin, transferrin, α_2-macroglobulin, ferritin, insulin, atrial natriuretic peptide, progesterone, estrogen, glucocorticoid, calcitonin, granulocyte colony stimulating factor 1, adenosine, β-adrenergic, endothelin, interferon-γ, epidermal growth factor, insulin-like growth factor, opioid or thromboxane receptors.

Receptors of the above categories may also be used to modify ligands or ligand analogues, or they may form part of the capturing agent, as indicated above.

Consequently, suitable ligands or ligand analogues may be selected from the group consisting of a growth factor, a peptide or polypeptide hormone, a cytokine, a detoxified toxin, a vitamin, a steroid hormone, or a substance acting on the central nervous system, e.g. a substance capable of binding to any one of the receptors listed above. Other possible ligands may be hyaluronan (cf. B. Smedsrød et al., op. cit.), chondroitin sulphate (cf. B. Smedsrød et al., op. cit.), N-terminal propeptide of collagen type III (cf. B. Smedsrød et al., op. cit.), mannose-terminated glycoconjugates (cf. B. Smedsrød et al., op. cit., or B. Smedsrød et al., Thromb. Haemostas. 59, 1988, pp. 480-484), a glycoside coupled to a bioactive substance capable of binding to the capturing agent

18

(the glycoside binding to the asialoglycoprotein receptor of hepatocytes) or tissue plasminogen activator. In certain special cases, one might contemplate using as the ligand a substance which does not bind to any specific receptor but

5 which is nevertheless bound to a specific organ (e.g. aprotinin which is bound in the kidneys).

The bioactive substance which may be either detected or eliminated by the method of the invention may be a drug

10 (including a narcotic drug), e.g. an alkaloid such as digoxin or morphine, or an antidepressive (including tricyclic antidepressives), acetylsalicylic acid, paracetamol, antiepileptic, β-receptor blocker, neuroleptic, sympatomimetic, a toxin, e.g. an exotoxin or endotoxin (LPS), snake venom,

15 fungal poison (e.g. muscarine), an inorganic poison such as a heavy metal (e.g. lead, cadmium, mercury), an organic poison such as an alkaloid (e.g. nicotine, scopolamine, atropine), a tumour-associated antigen, optionally as present on neoplastic cells, a substance produced by a neoplastic cell, an activated

20 leukocyte, an activated lymphocyte, a plasma cell, surface or circulating substances produced by cells, an antibody, in particular an autoantibody, a thrombus (or a component thereof) or a pathogen (e.g. virus, bacteria, fungi) or a component thereof, such as a surface antigen.

25

DESCRIPTION OF SPECIFIC EMBODIMENTS

I. Detection of vascular thrombi

30 Vascular thrombi may be rapidly detected by injecting an antibody which is specific for the seven first amino acids of the β-chain of human fibrin. These amino acids are not exposed on fibrinogen which implies that the antibody will react with fibrin, but not with fibrinogen (cf. K.Y. Huy et al., Science

35 222, 1983, pp. 1129-1132).

More specifically, this fibrin-specific antibody is prepared

by the method described in Huy et al., op. cit., and labelled with ^{111}In essentially as described by Hnatowich, J. Immun. Meth. 65, 1983, pp. 147-157. By injecting this antibody, any fibrin present in the circulation (e.g. deep venous thrombi or lung emboli) will be labelled with the antibody. After about one hour, a 2-10-fold molar excess of tissue plasminogen activator (tPA) conjugated with the fibrin β-chain heptapeptide mentioned above is then injected. This will result in rapid elimination of the background activity of the labelled antibody in the blood through the liver due to the binding of tPA to hepatic receptors, thereby improving the target-blood ratio of the labelled antibody to a significant degree.

II. Diagnosis of vascular thrombi

Vascular thrombi may also be detected by a method which is similar to the one described above, but in which a bispecific antibody reactive with the fibrin β-chain heptapeptide as well with tPA is injected and in which background activity is cleared by means of unmodified tPA.

More specifically, monoclonal antibodies reactive with the fibrin β-chain heptapeptide are prepared as described by Huy et al., op. cit. Monoclonal antibodies reactive with human tPA are prepared as described in DK 4082/89. Immunoreactive bispecific antibodies are prepared from the two monoclonal antibodies by the method described in EP 179 872 and labelled with ^{111}In essentially as described by Hnatowich, J. Immun. Meth. 65, 1983, pp. 147-157.

By injecting the bispecific antibody, any fibrin present in the circulation (e.g. deep venous thrombi or lung emboli) will be labelled with the antibody. After about one hour, a 2-10-fold molar excess of unmodified tPA (Actilyse®, available from Boehringer Ingelheim) is then injected. This will result in rapid elimination of the background activity of the labelled antibody in the blood through the liver due to the binding of

20

tPA to hepatic receptors, thereby improving the target-blood
ratio of the labelled antibody to a significant degree.

III. Localization of tumours

5

The localization of tumours by means of radiolabelled
antibodies is an established technique (cf., for instance,
Chatal, Monoclonal Antibodies in Immunoscintigraphy, CRC,
Florida, 1989). It is important to reduce the background
10   activity of the antibody (i.e. establish a high target-blood
ratio) in order to obtain a high sensitivity of the diagnosis.
Conventionally, this has been done by deferring tumour
detection until the level of circulating labelled antibodies
has diminished by natural elimination. The clearance of
15   labelled antibodies may be accellerated by injecting a
bispecific antibody reactive with a tumour-specific antigen as
well as with tPA, followed by injection of tPA after the
antibody has been bound to the tumour.

20   More specifically, monoclonal antibodies reactive with human
carcinoembryonic antigen (CEA) is prepared according to
standard procedures (cf. J.P. Mach et al., Immunology Today 2,
1981, pp. 239-242). Monoclonal antibodies reactive with human
tPA are prepared as described in DK 4082/89. Immunoreactive
25   bispecific antibodies are prepared from the two monoclonal
antibodies by the method described in EP 179 872. The
bispecific antibodies are labelled with ^{111}In essentially as
described by Hnatowich, J. Immun. Meth. 65, 1983, pp. 147-157.

30   The labelled bispecific antibodies are then injected into
patients with colorectal cancer and allowed to circulate for
about 30 minutes followed by injection of a 2-10-fold molar
excess of human tPA (Actilyse®, available from Boehringer
Ingelheim). This will result in rapid elimination of the
35   labelled antibody from the circulation to a level at which the
tumours are visualized clearly on the scintigrams (a level
below about 10-25% of the original level, as determined by

21

experiments with radiolabelled anti-CEA antibodies).

IV. Treatment of digoxin poisoning

5   Overdosing of digoxin leading to intoxication represents a
serious clinical problem because no efficacious antidote to the
drug exists at present. Parenteral administration of antibodies
specific for digoxin has previously been suggested in order to
reduce the free concentration of the drug (cf., for instance,
10   D.H. Smith and V.P. Butler, J. Clin. Invest. 50, 1971, pp.
1738-1744; W. Clarke and E.A. Ramoska, Am. J. Emerg. Med. 6(5),
1988, pp. 465-470). The clinical efficacy of such treatment
varies (S.S. Martiny et al., Crit. Care Med. 16(6), 1988, pp.
629-635). A major drawback of this treatment is that
15   potentially vital digoxin treatment cannot be initiated for
quite some time after injection of the antibody.

It may be assumed that more efficient elimination of the
circulating antibody-digoxin complex would more rapidly reduce
20   the free concentration of digoxin in the bloodstream. Such
elimination may be effected by initially injecting a bispecific
antibody reactive with digoxin as well as tPA in the
bloodstream of an intoxicated patient. After about 10-30
minutes, tPA is injected in a 2-10-fold molar excess of the
25   antibody. The complexes of digoxin, antibody and tPA (as well
as antibody and tPA not bound to digoxin) are then bound to the
tPA receptor in the liver (cf. D.C. Rijken et al., Thromb. Res.
57, Suppl. 10, 1990, pp. 63-71) and excreted therefrom.

30   More specifically, murine monoclonal antibodies reactive with
human tPA are produced by the method described in DK 4082/89.
Polyclonal antibodies against digoxin are prepared as described
by D.H. Smith and V.P. Butler, op. cit. Immunoreactive
bispecific antibodies are prepared from the two antibodies by
35   the method described in EP 179 872.

To test the theraputic efficacy of the method of the present

22

invention, four rabbits are treated with theraputic doses of digoxin (NEN, DuPont) for 5 days after which they are intoxicated by administrating an overdose of ^{3}H-labelled digoxin (NEN, DuPont). 6 hours later two of the rabbits are

5   treated with anti-digoxin antibody alone, while the other two rabbits receive an equivalent dose of the bispecific antibody followed by a 2-10-fold molar excess of tPA (e.g. Actilyse®, available from Boehringer Ingelheim) about 30 minutes later. Comparison of the radioactive counts of plasma samples taken

10   from each group of rabbits in the course of the treatment is expected to show a significant decrease of the plasma content of digoxin in the rabbits treated with the bispecific antibody and subsequently injected with tPA.

15   **V. Elimination of LPS from the circulation**

Endotoxin (LPS) plays a significant part in the pathogenesis of gram-negative septicemia (cf. T.B. Casale et al., _J. Allergy Clin. Immunol._ 85, 1990, pp. 45-51). It has previously been

20   attempted, with varying degrees of success, to neutralize LPS by administering polyclonal or monoclonal anti-LPS antibodies (cf. L.S. Young et al., _Rev. Infect. Dis._ 11, Suppl. 7, 1989, pp. S1564-S1571; B.J. Appelmelk et al., _Microb. Pathogen_ 5, 1988, pp. 251-257). A major obstacle to this approach is that

25   most anti-LPS antibodies do not protect against LPS toxicity. It is contemplated that elimination of LSP by the liver may be increased by administering a bispecific antibody which is reactive with LPS as well as with tPA, followed by injection of a suitable amount of tPA.

30

More specifically, monoclonal antibodies reactive with LPS are prepared according to standard procedures (cf. M.N. Nys et al., _J. Infect. Dis._ 162, 1990, pp. 1089-1095). Monoclonal antibodies reactive with human tPA are prepared as described

35   in DK 4082/89. Immunoreactive bispecific antibodies are prepared from the two monoclonal antibodies by the method described in EP 179 872.

The bispecific antibodies are then injected into septicemic patients and allowed to circulate for about 30 to 60 minutes to bind a significant proportion of the endotoxin present, followed by injection of a 2-10-fold molar excess of human tPA
5   (Actilyse®, available from Boehringer Ingelheim). This will result in a more rapid elimination of the LPS/antibody/tPA complex from the circulation.

BRIEF DESCRIPTION OF THE DRAWINGS
10

Fig. 1 is a graph showing the total radioactivity in plasma of In-111 labelled anti-t-PA monoclonal antibody (MoAb) with and without subsequent injection of t-PA.

15   Fig. 2 is a graph showing the immunoreactive part of In-111 labelled anti-t-PA MoAb with and without subsequent injection of t-PA.

Fig. 3 is a scintigram showing the organ distribution in
20   rabbits of In-111 labelled anti-t-PA MoAb alone and complexed with t-PA.

Fig. 4 is a graph showing the total radioactivity in human plasma of In-111 labelled anti-t-PA MoAb before and after
25   injection of t-PA.

Fig. 5 is a graph showing the change in human organ distribution of In-111 labelled anti-t-PA MoAb following injection of t-Pa.
30

Fig. 6 shows two scintigrams of the crural region initially showing increased accumulation of In-111 labelled anti-t-PA MoAb in the vascular bed of the right leg (A), and after t-PA injection revealing the focal thrombus area (top of the right
35   leg) (B).

Fig. 7 is a graph showing gelfiltration of a bispecific anti-t-

PA/anti-rabbit antibody. All fractions were tested for OD_{280} and reactivity in a t-PA enzyme immunoassay (EIA). All fractions were diluted 1:25000 before testing in the EIA system.

5   Fig. 8 is a graph showing the concentration, determined by EIA, of a bispecific anti-t-PA/anti-rabbit antibody in rabbit plasma with (indicated by squares) and without (indicated by triangles) injection of t-PA.

10 Fig. 9 is a graph showing the total amount, determined by EIA, of biotinylated bispecific anti-t-PA/anti-rabbit antibody in rabbit plasma with (indicated by squares) and without (indicated by triangles) injection of t-PA.

15 The present invention is illustrated in further detail in the following examples which are not in any way intended to limit the scope of the invention as claimed.

Example 1

20

Monofunctional capturing agent

(a) Production of a monoclonal antibody against t-PA

25 Purification of t-PA antigen

The t-PA used for the immunization of mice was purified as disclosed in Rijken, D.C. and Collen, D., _J. Biol. Chem_. 256, 1981, pp. 7035-41. The purified material contained >95% pure
30 t-PA as determined by silver-stained SDS-polyacrylamide gel electrophoresis.

Immunization of AKR mice with t-PA

35 Purified t-PA obtained as described above was dialysed against 0.15 M NaCl containing 0.01% (v/v) Tween 80, and the concentration was adjusted to 10 μg/ml.

AKR mice were immunized 3 times at bi-weekly intervals. For the
first two immunizations, each mouse was injected subcutaneously
with 50 μl t-PA emulsified with 50 μl of Freunds's incomplete
adjuvant (corresponding to 5 μg of t-PA/mouse). The last
5   immunization was identical to the first two, but was given
intraperitoneally rather than subcutaneously. Two months later
a mouse received an intravenous booster injection of 90 μl t-PA
without any Freund's adjuvant.

10   Cell fusion and culture of cells

Three days after the intravenous t-PA booster, the spleen was
removed, and a spleen cell suspension was prepared by carefully
dissecting and disrupting the spleen. The resulting spleen
15   cells were used for cell fusions as described in Petersen, L.C.
et al., Thrombosis and Haemostasis 57 (2), 1987, pp. 205-211.
In brief, spleen cells were fused with X63-Ag8-6.5.3 myeloma
cells in the presence of a polyethylene glycol solution. After
fusion the cells were seeded in 10 96-well microtiter cell
20   plates. Hybridoma supernatants were screened after two weeks
growth by enzyme-linked immunosorbent assay using t-PA as the
antigen. A positive clone was recloned several times by the
technique of limiting dilution to ensure monoclonality. The
cell line was then grown on a large scale in "cell factories"
25   from NUNC in a medium consisting of RPMI 1640 (Gibco, UK) with
2% BMS (Gibco, UK).

Purification of the monoclonal antibody

30   The hybridoma supernatants were concentrated. pH was adjusted
to 8.3, after which the monoclonal antibody was purified at 4°C
by adsorption to on a Protein A gel. Elution was performed with
a citrate buffer pH 4.5.

35   Production of F(ab)$_2$ fragments

The purified monoclonal antibody (subclass IgG$_1$) was digested

26

with pepsin at a enzyme/substrate ratio of 1:100 at pH 4.30 for 16 h at 37°C. The reaction was stopped by adjusting the pH of the reaction mixture to pH 8.5 with solid Tris. The F(ab)$_2$ was separated from undigested IgG and low molecular weight products on a Ultrogel AcA 44 column. Purification of the antibody, pepsin digestion and purification of F(ab)$_2$ was monitored on silver-stained SDS-gels.

Labelling of F(ab)$_2$ with indium

The purified F(ab)$_2$ was labelled with indium essentially according to the method of Hnatowich (J.Immun.Methods. **65**, 147-157 (1983).Immunoreactivity of conjugates was confirmed by ELISA and with immunosorbent technique using t-PA as antigen.

The immunochemical purity of the labelled antibody was determined as the percentage of antibody radioactivity capable of binding to a t-PA coupled Sepharose (Pharmacia AB, Sweden) column. The immunochemical purity of the conjugate was less than the radiochemical purity since some of the labelled antibody was immunological by non-reactive. The immunochemical purity of the antibody was 93%.

(b) Biological background subtraction - animal study

t-PA

The t-PA used in the experiments was Actilyse obtained from Boehringer Ingelheim.

Experimental design

Two rabbits were injected intravenously with the antibody in dose/kg and specific radioactivity comparable to the human studies mentioned in Example 2.

Rabbit 1 received antibody at t = 0 minutes. No t-PA was subsequently administered. Rabbit 2 received antibody at t = 0 minutes. At t = 120 minutes rabbit 2 received an intravenous injection of t-PA administered over a period of 1 minute. The
5 injected amount of t-PA was in 3 x molar concentration of the administered antibody.

Plasma activity

10 Blood samples were taken at t = 0, 10, 20, 40, 60 min and 2, 4, 6, 12, and 24 hours after the antibody injection. In rabbit 2 additional samples were taken 5, 10, 20, 40, and 60 min after the injection of t-PA.

15 For each sample, whole plasma activity was counted, and by regression analysis (Freelance Plus ver 3.01) these data were adjusted to an exponential curve and the overall biological half-life was calculated. All curves presented were corrected for physical decay. The results are shown in Fig. 1.
20

$T\frac{1}{2}$ of the antibody was 10.3 h.

When t-PA was added at t = 2 hours a dramatic decrease in plasma activity was observed. Within 10 min a 92% reduction was
25 observed.

The biological half-life of the radiolabelled immunoreactive antibody in plasma was determined as the half-life of t-PA binding activity. The results are shown in Fig. 2.
30

Within 10 minutes after the injection of t-PA at t = 2 hours more than 99% of the immunoreactive antibody activity was eliminated from the plasma. The half-life of this elimination is 1 minute. A slight increase in activity - up to 1.6% - is
35 seen in the hours after the initial elimination. This is undoubtedly caused by redistribution of the extravascular antibody pool.

28

Whole body activity

After 24 hours the rabbits were sacrificed. Whole body
scintigrams of the rabbits were obtained. The results are shown
5   in Fig. 3. Antibody not complexed with t-PA was mainly located
in the kidneys, whereas antibody complexed with t-PA was almost
exclusively found in the liver.

It appears from these experiments that t-PA/anti-t-PA MoAb
10   complexes have a substantially shorter half-life in the
organism than anti-t-PA alone. Furthermore, the experiments
show that is is possible to exploit the rapid t-PA/anti-t-PA
MoAb clearance in vivo. The results of the study suggest that
the hepatic t-PA receptor is responsible for this increase in
15   antibody clearance.

The decline in total radioactivity is an essential parameter
for imaging, in that the total plasma activity will decide the
target/blood ratio. The reduction in t-PA binding radioactivity
20   is a measure for the in vivo efficiency of the receptor
elimination pathway. The decrease in total radioactivity will
be less than the reduction in t-PA binding radioactivity unless
the conjugate is 100% immunochemically active.

25   ## Example 2

Biological background substraction - human study

A 77 year-old woman was admitted to hospital because of
30   pronounced oedema of the right leg. An acute contrast
phlebography exposed signs of a thrombosis in one of the
lateral crural veins of the right leg. Scintigraphy was
performed using a radiolabelled monoclonal antibody prepared
as described in example 1. The radiochemical purity was only
35   67%.

One hour after injection of the radiolabelled antibody 5 mg of

t-PA (Actilyse Boehringer Ingelheim) was injected.

Plasma activity

5   Blood samples were taken at regular intervals after the t-PA
injection. For each sample, whole plasma radioactivity was
counted in a well gamma-counter and plotted against time (Fig.
4). Exponential regression analysis was performed on Freelance
Plus ver 3.01). T½ of the initial phase was 4.8 h. After t-PA
10 injection a substantial decrease in plasma activity was
observed. Within 17 min a 50% reduction was observed.

Scintigraphy

15 Biodistribution:

During t-PA injection a dynamic acquisition of the liver and
heart region was performed on the gamma-camera with 60 frames
lasting 15 sec each. Activity of the blood (heart region) and
20 the liver were calculated and plotted against time. The shift
in distribution from circulating blood activity to bound liver
activity following injection is clearly demonstrated on Fig.
5.

25 Thrombus detection:

Initially, signs of stasis in the lower leg were revealed.
However, the precise location of the thrombus could not be
detected. After the t-PA injection, the activity in the dilated
30 vascular bed diminished, and the thrombus area was detected
(Fig. 6).

The biological background subtraction appears to improve the
imaging technique, making it possible to detect thrombosis
35 within a few hours.

Example 3

Bifunctional capturing agent

(a) Production of a monoclonal antibody against t-PA

5   Purification of t-PA antigen

The t-PA used for the immunization of mice was purified as
disclosed in Rijken, D.C. and Collen, D., J. Biol. Chem. 256,
1981, pp. 7035-41. The purified material contained >95% pure
10 t-PA as determined by silver-stained SDS-polyacrylamide gel
electrophoresis.

Immunization of RBF/Dn mice with t-PA

15 Purified t-PA obtained as described above was dialysed against
0.15 M NaCl containing 0.01% (v/v) Tween 80, and the concentra-
tion was adjusted to 10 μg/ml.

Origin of parent myeloma cell line:

20
The myeloma cells used for production of the monoclonal anti-
body 3 F1 were the FOX-NY myeloma line deficient in the selec-
table enzyme marker locus adenosine phosphoribosyl transferase
(ARPT⁻) and hypoxanthine phosphoribosyl transferase (HRPT⁻).
25 This myeloma cell line is "non-producing" cell line, ie. it
does not secrete or synthesize any immunglobulin chains.

Origin of animal strain

30 RBF/Dn-strain mice (obtained from the Jackson Laboratory, Bar
Harbor, Maine, USA) containing the RB (8.12)5Bnr Robertsonian
translocation chromosome were used for production of monoclonal
antibodies.

35 Immunization and fusion

RBF/Dn-strain mice were immunized three times at bi-weekly

intervals with purified human t-PA. Antigen was emulsified 1:1 in Freund's incomplete adjuvant prior to immunization of the mice. Each mouse received 10 μg antigen with each immunization in a total volumen of 200 μl. One month after the last immuni-
5   zation a mouse was boosted intravenously with 10 μg human t-PA. After another three days the mouse was sacrificed and the spleen removed for fusion with myeloma cells.

Spleen cells from one mouse (10.2 x 10^7 cells) were fused with
10 2.5 x 10^7 cells of the FOX-NY cell line. The fused cells were seeded on BALB/strain mouse macrophage feeder cells in a total of 10 96-well microtiter plates in a medium consisting of RPMI-1640 with 15 % v/v fetal calf serum (Gibco of Flow) supple-mented with adenine (7.5 x 10^{-5} M), hypoxanthine (8 x 10^{-4} M,
15 aminopterin (8 x 10^{-7} M) and thymidine (1.6 x 10^{-5} M) (AHAT). Consequently, the exposure of cell fusion mixture to a medium requiring ARPT activity (ARPT⁺ selection) eliminates both unfused ARPT⁻ myelomas and ARPT⁻ hybridomas. This results in increased stability of immunoglobulin producing hybridomas
20 (R.T.Taggart and I.M.Samloff: Science 219 (1983) pp. 1228 - 1230).

Hybridoma supernatants were screened after two weeks' growth
25 by enzyme linked immunosorbent assay using t-PA as the antigen. A positive clone was recloned several times by the technique of limiting dilution to ensure monoclonality. The cell line was then grown on a large scale in "cell factories" from NUNC in a medium consisting of RPMI 1640 (Gibco, UK) with 2% BMS
30 (Gibco, UK).

Purification of the monoclonal antibody

The hybridoma supernatants were concentrated. The pH was ad-
35 justed to 8.3, after which the monoclonal antibody was purified at 4°C by adsorption to on a Protein A gel. Elution was performed with a citrate buffer pH 4.5.

32

Production of F(ab)₂ fragments

The purified monoclonal antibody (subclass IgG_1) was digested
with pepsin at a enzyme/substrate ratio of 1:100 at a pH of
5   4.10 for 16 h at 37°C. The reaction was stopped by adjusting
the pH of the reaction mixture to pH 8.5 with solid Tris. The
$F(ab)_2$ was separated from undigested IgG on a Protein A column
and further purification was performed on a Ultrogel AcA 44
column. Purification of the antibody, pepsin digestion and
10 purificaton of $F(ab)_2$ was monitored on Coomassie-stained SDS-
gels.

Purification of rabbit immunoglobulin

15 Rabbit serum was adjusted to a pH of 7.5, after which the
rabbit immunoglobulin was purified at 4°C by adsorption to on
a Protein A gel. Elution was performed with a citrate buffer
pH 3.5.

20 ## Production af rabbit F(ab)₂ fragments

The purified rabbit immunoglobulin was digested with pepsin at
a enzyme/substrate ratio of 1:100 at pH 4.50 for 16 h at 37°C.
The reaction was stopped by adjusting the pH of the reaction
25 mixture to pH 8.5 with solid Tris. The $F(ab)_2$ was separated
from undigested IgG on a Protein A column and further
purification was performed on a Ultrogel AcA 44 column.
Purification of the antibody, pepsin digestion and purification
of $F(ab)_2$ was monitored on Coomassie-stained SDS-gels.

30

Biotinylation of rabbit immunoglobulin

The purified rabbit $F(ab)_2$ was biotinylated with Biotinyl-ε-
aminocaproic acid N-hydroxysuccinamidester (biotin-x-NHS)-
35 (Calbiochem 203188). Immediately prior to use, biotin-x-NHS is
dissolved in DMSO. The biotinylation was performed in a PBS
buffer pH 7.2 at 20°C with a molar ratio of biotin-x-NHS/$F(ab)_2$

of 100. The biotinylation process was stopped after 2 hours by
addition of 10 % v/v of imidazol buffer, 0.5 M pH 7.3.

Preparation of a bifunctional F(ab)₂

Preparation of the bifunctional antibody was performed
essentially according to Glennie, M.J. et al. J.Immunol. vol
139, page 2367-2375 1987.

Reduction of biotinylated rabbit F(ab)₂

1 ml purified biotinylated rabbitF(ab)₂ at a concentration of
10 mg/ml was reduced with 10 mM DTE flushed with nitrogen and
stored for 60 minutes at 20°C followed by 30 minutes at 4°C.
Separation af Fab from DTE was performed on a 1.5x15 cm
Sephadex G-25 column equilibrated with 50 mM acetate buffer ph
5.3 with 2 mM EDTA. The eluted Fab was pooled and stored on ice
before use.

Reduction and maleimide coupling of murine F(ab)₂

1 ml purified monoclonal anti t-PA F(ab)₂ at a concentration of
10 mg/ml was reduced with 10 mM DTE, flushed with nitrogen and
stored for 60 minutes at 20°C followed by 30 minutes at 4°C.
Separation af Fab from DTE was performed on a 1.5x15 cm
Sephadex G-25 column equilibrated with 50 mM acetate buffer pH
5.3 containing 2 mM EDTA. The eluted Fab was pooled. To 3.2 ml
Fab at a concentration of 1.55 mg/ml was added 1.6 ml 12 mM mM
o-phenylene dimaleimide (o-PDM, SIGMA) dissolved in chilled
dimethylformamide. After 30 minutes the maleidated Fab$_{mal}$ was
separated from the other solutes in the reaction mixture by
passage through a 2.5x 15 cm Sephadex G-25 column equilibrated
with 50 mM sodium acetate pH 5.3 containing 2 mM EDTA.

Coupling of murine Fab$_{mal}$ with biotinylated rabbit Fab

x₁ ml Fab$_{mal}$ at a concentration of 0.41 mg/ml (82 nmol) was

mixed with 3.3 ml biotinylated rabbit Fab at a concentration
of 1.62 mg/ml (107 nmol). The reaction mixture was concentrated
to 2 mg/ml by ultrafiltration under nitrogen using a Amicon YM-
10 membrane in a chilled Amicon chamber (Amicon Ltd).

5

Gelfiltration of bispecific antibody

The reaction mixture was gelfiltrated an a 1.5x100 cm Ultrogel
AcA44 column equilibrated with 0.2 M phosphate buffer pH 6.0
10 containing 5 mM EDTA. The flow rate was 8 ml/h and the
fractions were of 1 ml.

EIA test of gelfiltrated bispecific antibody

15 All fractions were assayed by EIA. 96-well plates were coated
with human t-PA at a concentration of 1 µg/ml for 16 hours at
room temperature. After blocking the plates, all fractions were
assayed in this EIA. Fractions were tested in dilutions of
1:500 and 1:25000 in a PBS buffer containing 0.02% Tween 20
20 supplemented with 1 % BSA. Plates were washed after incubation
for 1 hour. The plates were then incubated with streptavidin-
peroxidase (Kirkegård and Perry Laboratories, Inc) in a
dilution of 1:20000 in a PBS buffer supplemented with 0.5 M
NaCl. After a final washing, the wells were incubated with 100
25 µl substrate buffer (citrate perborate buffer, pH 5.5,
containing ortho-phenylenediamine, 0.8 mg/ml) for 5 minutes,
after which the reaction was stopped by addition of 100 µl 2
M sulfuric acid to each well. Plates were read at 492 nm.

30 Results are shown in Fig. 7. In this figure, absorbance in the
EIA of all fractions diluted 1/x is depicted.

Fractions containing bispecific antibodies as estimated by the
EIA were pooled. The volume was 6 ml and the antibody
35 concentration (determined as A_{280}) was 198 µg/ml. This pool af
bispecific antibody was used for elimination experiments.

(b) Biological background subtraction

<u>t-PA</u>

5   The t-PA used in the experiments was Actilyse obtained from
Boehringer Ingelheim.

<u>Experimental design</u>

10   Four rabbits were injected intravenously with the bispecific
antibody in a dose of 50 µg/rabbit.

Rabbit 1 and 2 received antibody at t = 0 minutes. No t-PA was
subsequently administered. Rabbit 3 and 4 received antibody at
15   t = 0 minutes. At t = 60 minutes rabbit 3 and 4 received an in-
travenous injection of t-PA administered over a period of 1
minute. The injected amount of t-PA was in 12 x molar
concentration of the administered antibody.

20   <u>Plasma activity</u>

Blood samples were taken at t = 0, 10, 20, 40, 60 min and 2,
4, 6 after the antibody injection. In rabbit 3 and 4 additional
samples were taken 5, 10, 20, 40, and 60 min after the
25   injection of t-PA.

The concentration of bispecific antibody was determined in the
above mentioned EIA using a calibration curve (200 ng/ml to
0.78 ng/ml) of bispecific antibody in rabbit plasma. All
30   samples were tested undiluted and diluted 1:10 in normal rabbit
plasma.

The results are shown in Fig. 8 in which each curve represents
the mean value of two rabbit plasma samples. The half-life of
35   the bispecific antibody in the control rabbits that did not
receive any t-PA was approximately 4.5 hours. When t-PA was
added at t = 1 hours a dramatic decrease in the serum

concentration of the bispecific antibody was observed. Within
10 min substantially all of the bispecific antibody had been
cleared from the bloodstream as evaluated by the EIA results.

5   The results were verified in an EIA which measured the total
amount of biotinylated antibody using a microtiter plate in
which the wells were coated with streptavidin. The
concentration of bispecific antibody in rabbit plasma was
determined in this EIA using a calibration curve (200 ng/ml to
10   0.78 ng/ml) of bispecific antibody in rabbit plasma. The
response of this calibration curve was unaffected by the
addition of high concentrations of t-PA. Samples were incubated
undiluted and diluted 1:10 in normal rabbit plasma. CAlibrators
and samples were incubated for 1 hour. The plates were then
15   incubated with streptavidin-peroxidase (Kirkegård and Perry
Laboratories, Inc) in a dilution of 1:20000 in a PBS buffer
supplemented with 0.5 M NaCl. After a final washing, the wells
were incubated with 100 μl substrate buffer (citrate perborate
buffer pH 5.5 supplemented with ortho-phenylenediamine 0.8
20   mg/ml) for 5 minutes, after which the reaction was stopped by
addition of 100 μl 2 M sulfuric acid to each well. Plates were
read at 492 nm.

The results of are shown in Fig. 9 in which each curve
25   represents the mean value of two rabbit plasma samples. All
antibody concentrations are given as percentages, antibody
concentrations measured after 10 minutes being set at 100%. The
half-life of the bispecific antibody in control rabbits that
dis not receive any t-PA was approximately 4 hours. Within 10
30   minutes after the administration of t-PA, a decrease in the
concentration of biotinylated bispecific antibody of
approximately 80% was observed.

The results of this experiment show that the elimination of
35   bispecific antibody as shown in Fig. 9 is not simply caused by
a reaction between the bispecific antibody and t-PA making the
compleks unable to bind to the solid phase t-PA in the EIA

plate.

It appears from these experiments that t-PA/anti-t-PA bi-
specific antibodies have a substantially shorter half-life in
5 the organism than bispecific antibodies alone. Furthermore, the
experiments show that is is possible to exploit the rapid t-
PA/anti-t-PA MoAb clearance in vivo.

38

CLAIMS

1. A pharmaceutical or diagnostic composition comprising, in separate containers,

5

(a) a capturing agent capable of binding to a bioactive substance as well as to a ligand or ligand analogue which is able to bind to a cellular receptor; and

10 (b) a ligand or ligand analogue capable of binding to a cellular receptor as well as to said capturing agent.

2. A composition according to claim 1, wherein the capturing agent is an agent to be cleared rapidly from the circulation.

15

3. A composition according to claim 2, wherein the capturing agent is monofunctional, and wherein the ligand or ligand analogue is modified so as to be able to bind to the capturing agent.

20

4. A composition according to claim 2, wherein the capturing agent is monofunctional, and wherein the bioactive substance and the ligand are substantially identical.

25 5. A composition according to claim 1, wherein the bioactive substance is a substance to be cleared rapidly from the circulation.

6. A composition according to claim 5, wherein the capturing
30 agent is monofunctional, and wherein the bioactive substance is different from the ligand or ligand analogue which is modified so as to be able to bind to the capturing agent.

7. A composition according to claim 5, wherein the capturing
35 agent is bifunctional, and wherein the bioactive substance is different from the ligand or ligand analogue.

REPLACEMENT SHEET

8. A composition according to claim 7, wherein the ligand or ligand analogue is modified so as to be able to bind to the capturing agent.

5 9. A composition according to claim 2 or 4, wherein the capturing agent is monofunctional and comprises an antibody or a cellular receptor (other than the specific ligand-binding receptor) or a fragment thereof including the binding site for the bioactive substance.

10

10. A composition according to claim 3 or 6, wherein the capturing agent is monofunctional, and wherein the ligand or ligand analogue is modified so as to comprise an antibody (including an anti-idiotype antibody), a cellular receptor

15 (other than the specific ligand-binding receptor) or a fragment thereof including the binding site for the capturing agent, or wherein the ligand or ligand analogue is modified with a bioactive substance or a derivative or fragment thereof capable of binding the capturing agent.

20

11. A composition according to claim 7, wherein the capturing agent is bifunctional and comprises

(a) a bispecific antibody,

25

(b) a conjugate of an antibody and a cellular receptor (other than the specific ligand-binding receptor) or a fragment thereof comprising the binding site for the bioactive substance, a single-stranded oligonucleotide, an intercellular

30 adhesion molecule, or a complexing agent (e.g. biotin, avidin, lectin or a drug), or

(c) a conjugate of a cellular receptor (other than the specific ligand-binding receptor) or a fragment thereof comprising the

35 binding site for the bioactive substance and a single-stranded oligonucleotide, an intercellular adhesion molecule, or a complexing agent (e.g. biotin, avidin, lectin or a drug).

12. A composition according to claim 11, wherein the ligand or ligand analogue is modified so as to comprise an antibody, a cellular receptor (other than the ligand-binding receptor) or a fragment thereof comprising a binding site for the capturing
5 agent, a single-stranded oligonucleotide, an intercellular adhesion molecule or a complexing agent, each of which is capable of forming a conjugate with the part of the capturing agent which does not bind the bioactive substance.

10 13. A composition according to any of claims 1-12, wherein the agent is provided with a label.

14. A composition according to claim 13, wherein the label is selected from the group consisting of radioactive isotopes,
15 magnetite particles, and paramagnetic atoms.

15. A composition according to any of claims 1-14, wherein the cellular receptor is a liver, spleen, bone marrow, lung, kidney, skin or placental receptor.
20
16. A composition according to any of claims 1-15, wherein the bioactive substance is a drug (including a narcotic drug), e.g. an alkaloid such as digoxin or morphine, or an antidepressive (including tricyclic antidepressives), acetylsalicylic acid,
25 paracetamol, antiepileptic, β-receptor blocker, neuroleptic, sympatomimetic, a toxin, e.g. an exotoxin or endotoxin (LPS), snake venom, fungal poison (e.g. muscarine), an inorganic poison such as a heavy metal (e.g. lead, cadmium, mercury), an organic poison such as an alkaloid (e.g. nicotine, scopolamine,
30 atropine), a tumour-associated antigen, optionally as present on neoplastic cells, a substance produced by a neoplastic cell, an activated leukocyte, an activated lymphocyte, a plasma cell, surface or circulating substances produced by cells, an antibody, in particular an autoantibody, a thrombus (or a
35 component thereof) or a pathogen (e.g. virus, bacteria, fungi) or a component thereof, such as a surface antigen.

41

17. A composition according to any of claims 1-16, wherein the ligand or ligand analogue is a growth factor, a peptide or polypeptide hormone, a cytokine, a detoxified toxin, a vitamin, a steroid hormone, a substance acting on the central nervous

5   system, hyaluronan, , chondroitin, sulfate, N-terminal peptide of collagen type III, mannose-terminated glycoconjugates, a glycoside (coupled to a bioactive substance) or tissue plasminogen activator.

10 18. A method of providing the rapid clearance of a bioactive substance from the circulation, the method comprising administering, to a subject in need hereof,

(a) an effective amount of a capturing agent capable of binding
15 a bioactive substance as well as a ligand or ligand analogue which is able to bind to a cellular receptor,

followed, after a suitable interval, by administration of

20 (b) an effective amount of a ligand or ligand analogue capable of binding to a cellular receptor as well as to said capturing agent.

19. A method of rapid _in vivo_ detection of a pathological
25 condition, the method comprising administering

(a) an effective amount of a capturing agent capable of binding a bioactive substance characteristic of the pathological condition in question as well as a ligand or ligand analogue
30 which is able to bind to a cellular receptor,

followed, after a suitable interval, by administration of

(b) an effective amount of a ligand or ligand analogue capable
35 of binding to a cellular receptor as well as to said capturing agent so as to provide a rapid clearance of the capturing agent from the circulation.

42

20. Use of a capturing agent capable of binding a bioactive
substance as well as a ligand or ligand analogue which is able
to bind to a cellular receptor for the manufacture, in
combination with a ligand or ligand analogue capable of binding
5   to a cellular receptor as well as to said capturing agent, of
a pharmaceutical composition providing the rapid clearance of
a bioactive substance from the circulation.

21. Use of a capturing agent capable of binding a bioactive
10 substance characteristic of a pathological condition as well
as a ligand or ligand analogue which is able to bind to a
cellular receptor for the manufacture, in combination with a
ligand or ligand analogue capable of binding to a cellular
receptor as well as to said capturing agent, of a diagnostic
15 composition providing the rapid clearance of the capturing
agent from the circulation and hence a rapid detection of the
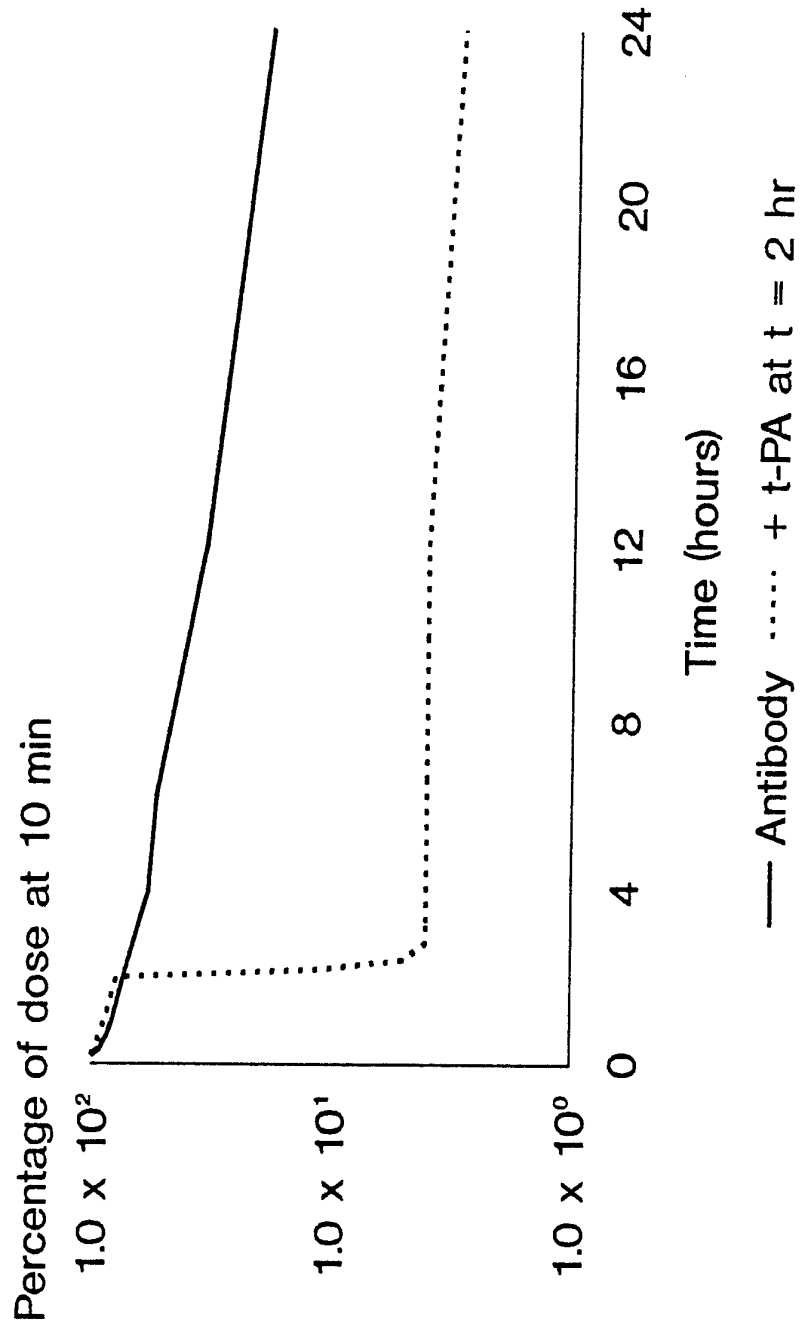pathological condition in question.

Total activity in plasma

Percentage of dose at 10 min

1.0×10^2

1.0×10^1

1.0×10^0

0　　4　　8　　12　　16　　20　　24

Time (hours)

—— Antibody　······ + t-PA at t = 2 hr

Fig. 1
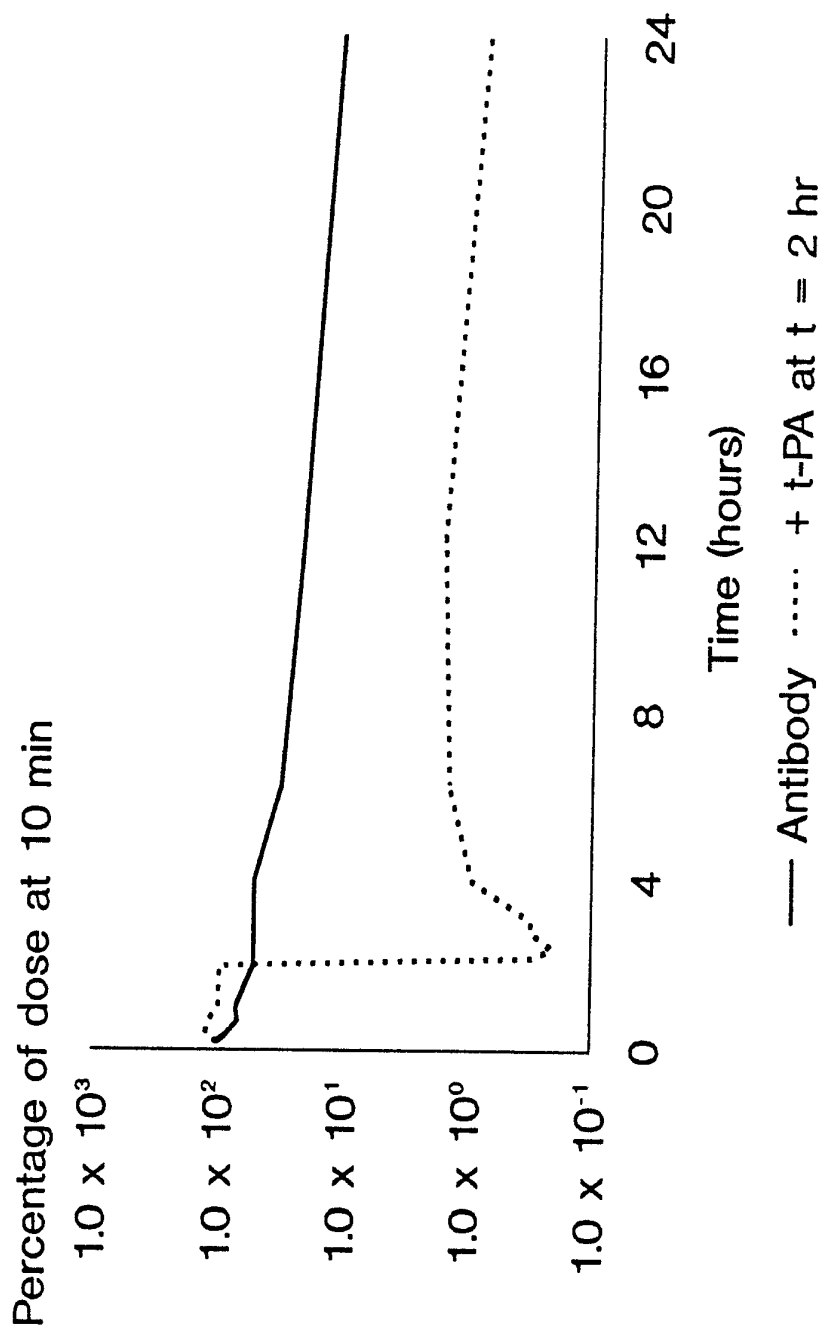
Fig. 2

3/9

RABBITS

HEAD

ANTERIOR VIEW

ANTIBODY   ANTIBODY + T-PA
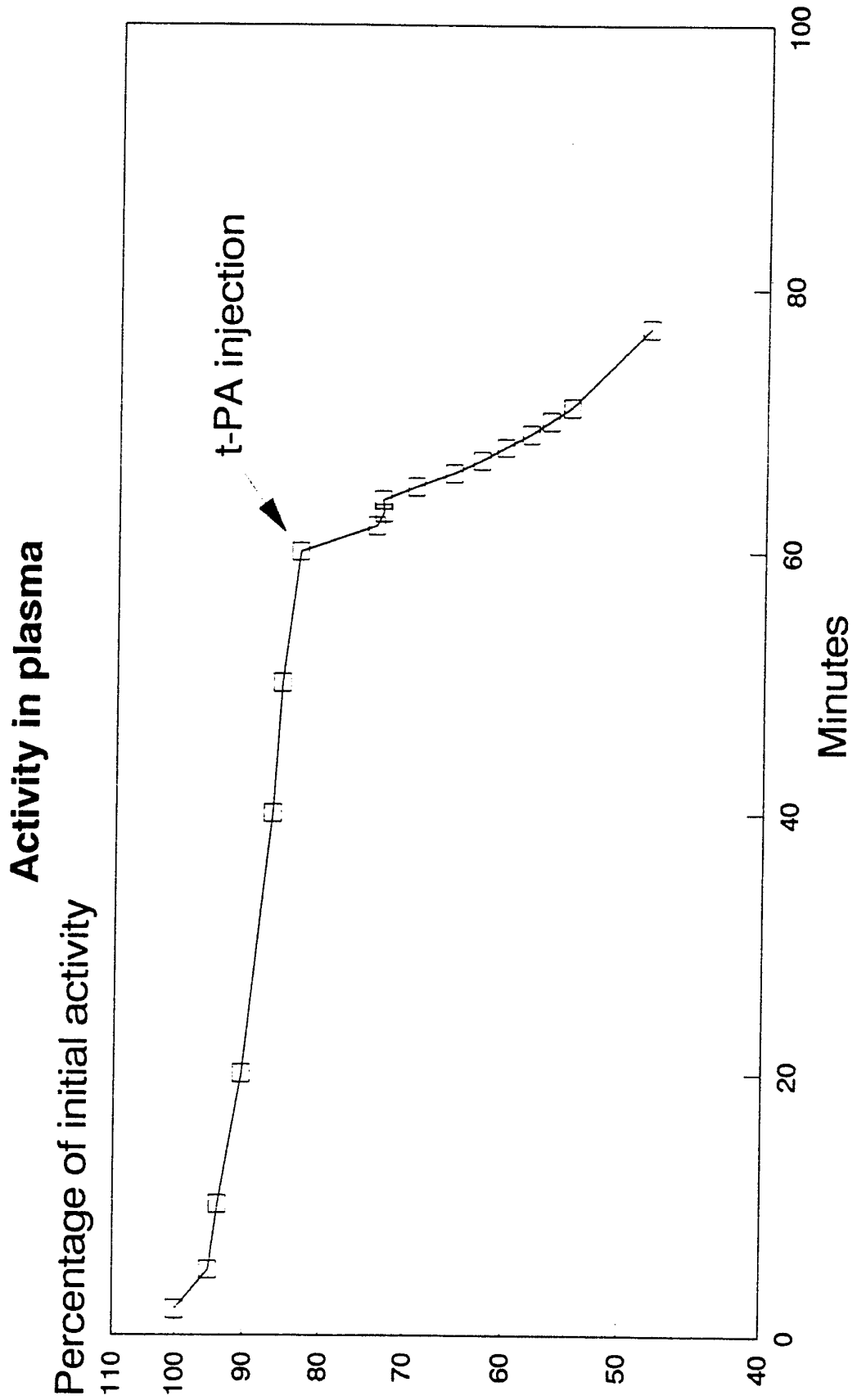
Fig. 3

Fig. 4

Fig. 5

BEFORE T-PA INJECTION.   AFTER T-PA INJECTION.

CRURAL REGION - ANTERIOR VIEW.

Fig. 6

Fig. 7

8/9

t-PA-EIA

Percentage of dose at 10 min

Fig. 8

Avidin-EIA

Percentage of dose at 10 min

Time (minutes)

F2   F2 + t-PA
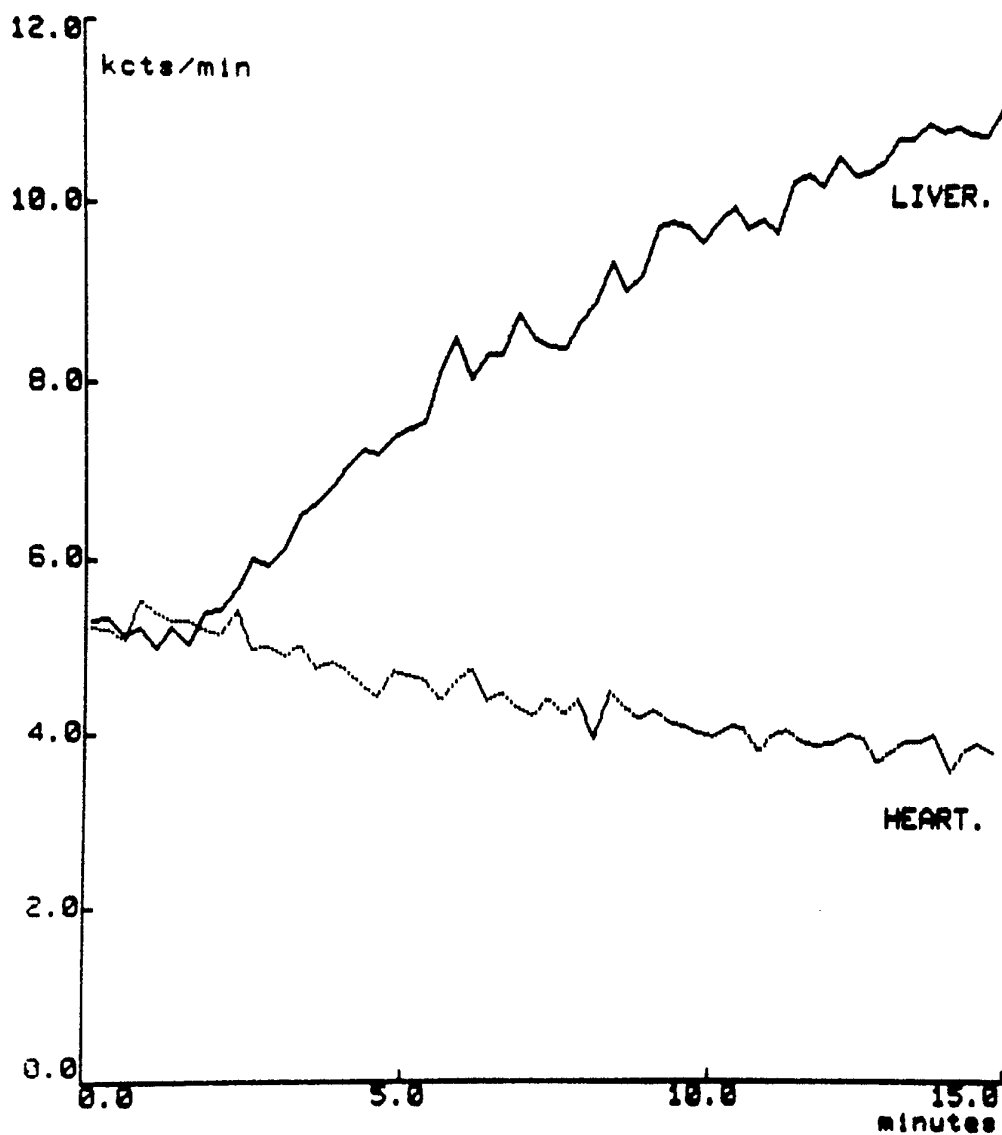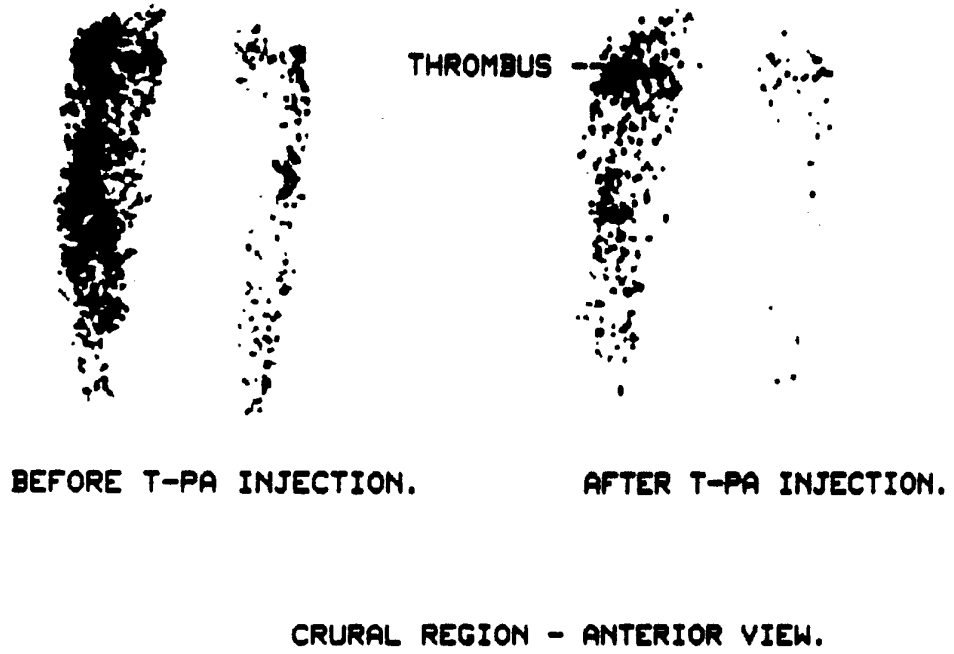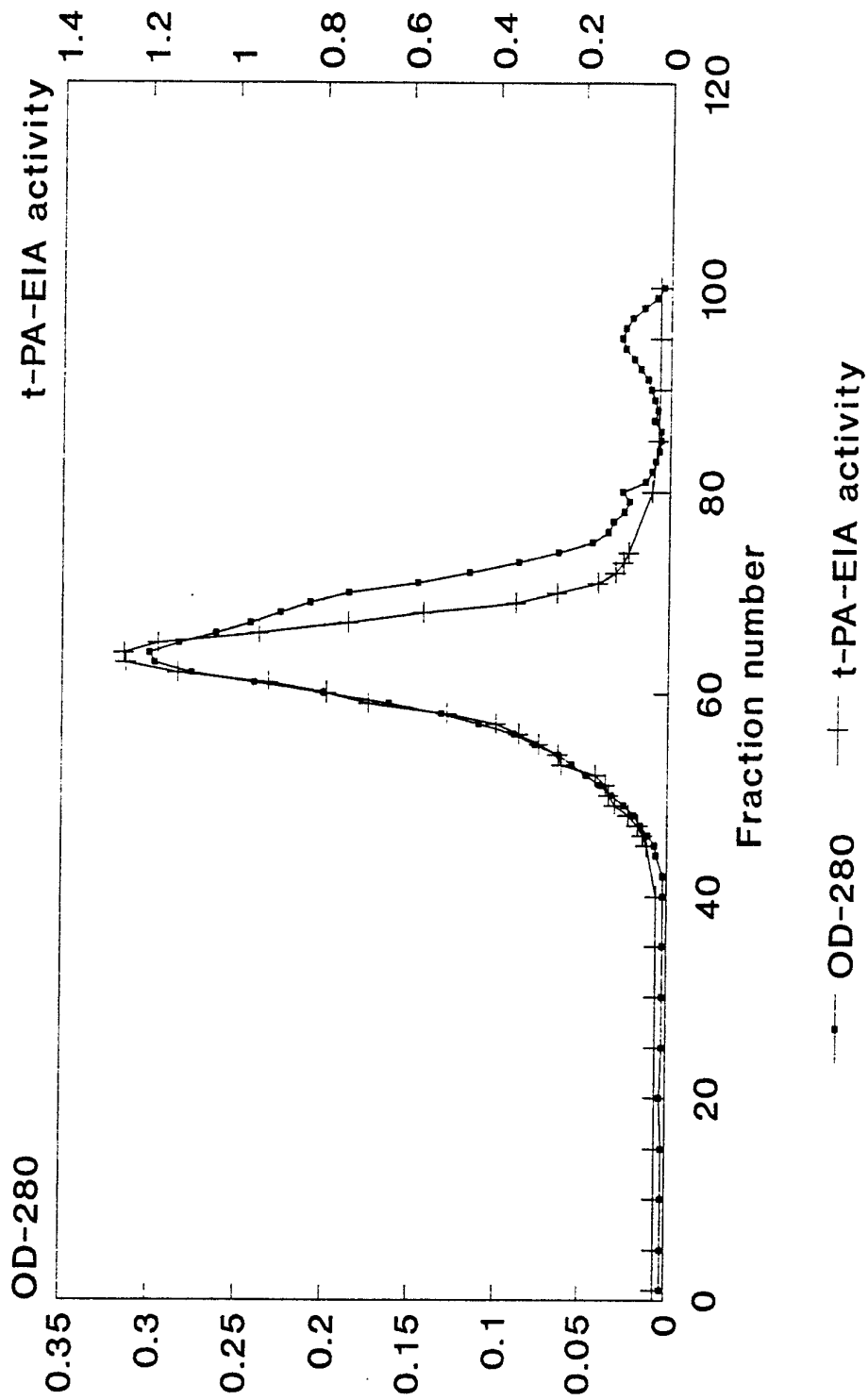
Fig. 9

INTERNATIONAL SEARCH REPORT

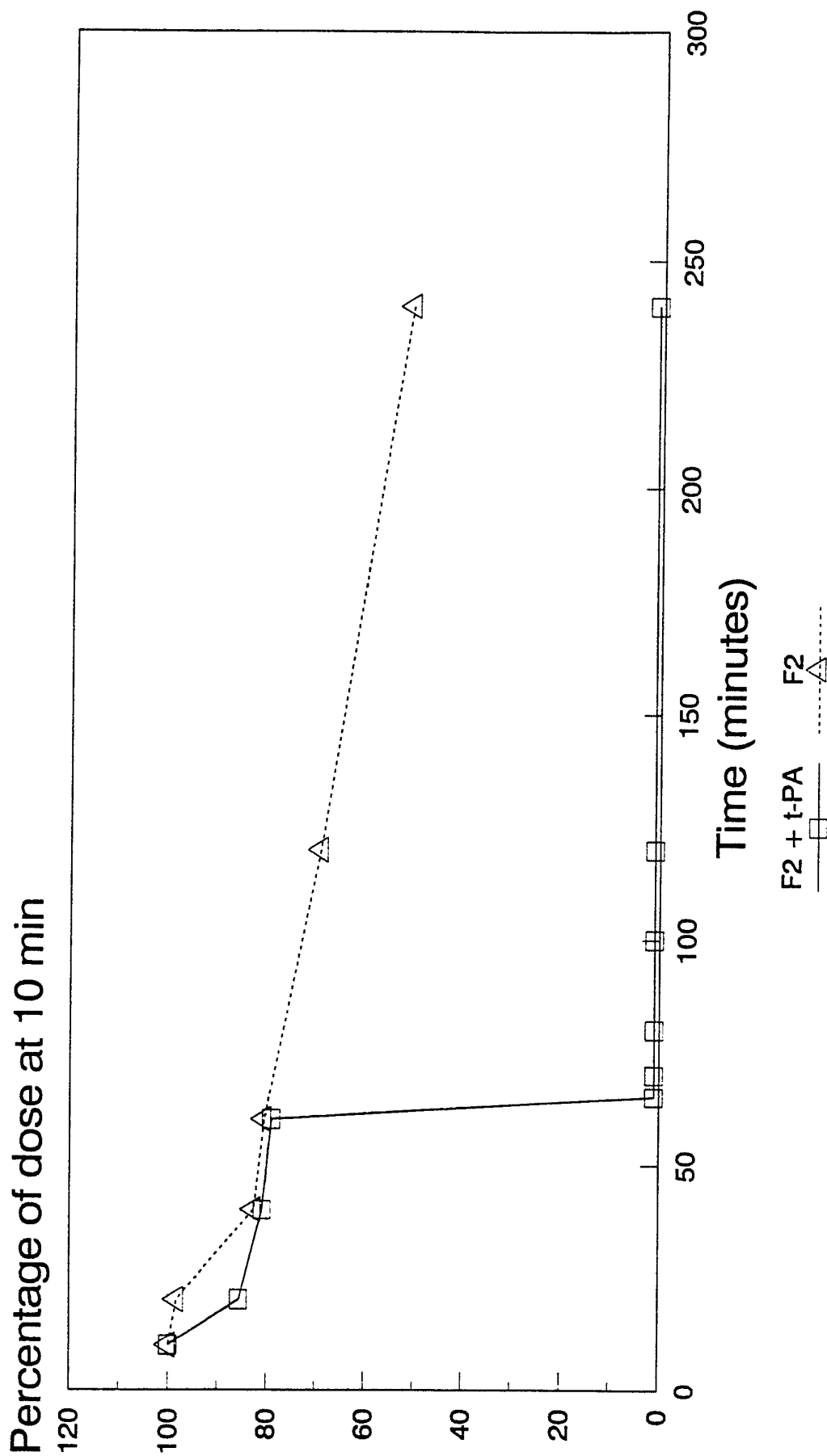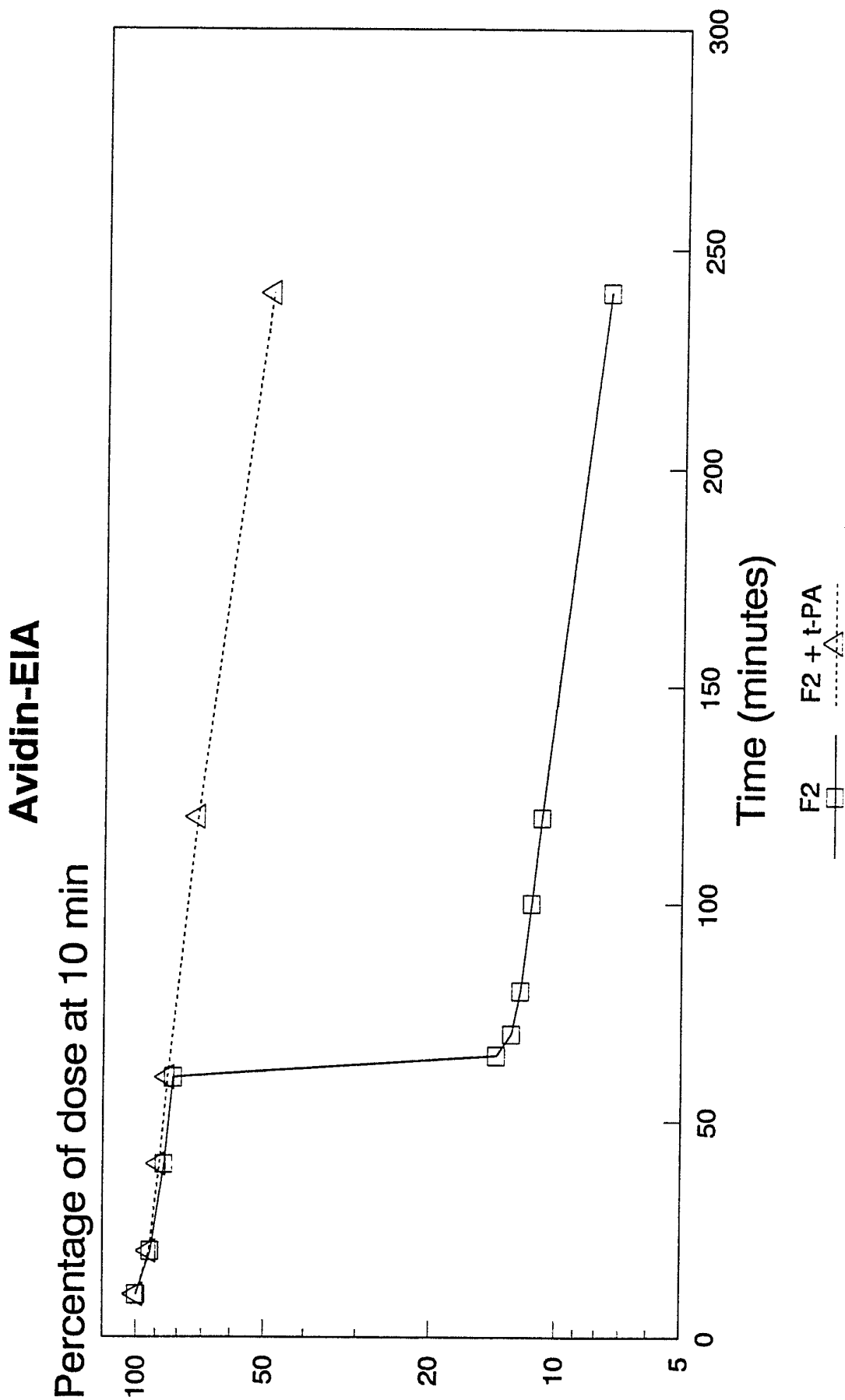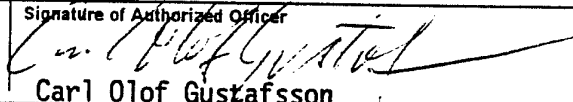International Application No **PCT/DK 91/00215**

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: A 61 K 39/00, 47/48, 49/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched[8]

SE,DK,FI,NO classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category*	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	EP, A1, 0308208 (CENTER FOR MOLECULAR MEDICINE AND IMMUNOLOGY) 22 March 1989, see in particular page 2, line 28-41	1,2,5,7, 11-17, 20,21
Y		1-17,20, 21
	--	
X	WO, A1, 8905140 (BRADWELL, ARTHUR R) 15 June 1989, see in particular pages 8-10	1,2,5,7, 11-17, 20,21
Y		1-17,20- 21
	--	
Y	Patent Abstracts of Japan, Vol 7, No 210, P223, abstract of JP 58-103664, publ 1983-06-20 Teijin K.K.	1-17,20, 21
	--	

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
31st October 1991	1991 -11- 0 4

International Searching Authority	Signature of Authorized Officer
SWEDISH PATENT OFFICE	Carl Olof Gustafsson

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No

X	Biochemical society transactions, Vol. 18, 1990
	    K.D. Bagshawe: "Antibody-directed
	    enzyme/prodrug therapy (ADEPT)",
	    see page 750 - page 752
	    see p 751 left column "A three-stage
	    system" and "Discussion"
	    --
		1,2,5,7,
		11-17,
		20,21

X
 	
Y
	US, A, 4863713 (GOODWIN D A ET AL.)
	    5 September 1989,
	    see the whole document
	    --
		1,2,5,7,
		11-17,
		20,21
		1-17,20,
		21

Y	EP, A2, 0353960 (IMMUNOMEDICS, INC.)
	    7 February 1990, see column 15,
	    line 31 - column 17, line 37
	    --
		1-10

A	US, A, 4932412 (GOLDENBERG M D) 12 June 1990,
	    see column 2-3; column 12, line 49 -
	    column 13, line 41
	    --
		1-10

A	EP, A1, 0149709 (GOLDENBERG M D)
	    31 July 1985,
	    see the whole document
	    --
		1-10

A	US, A, 4624846 (GOLDENBERG M D) 25 November 1986,
	    see the whole document
	    --
		1-10

Y	Science, Vol. 243, June 1989 E Haber et
	    al.: "Innovative Approaches to Plasminogen
	    Activator Therapy", see page 51 - page 56
	    see pages 55-56
	    --
		1-17,20,
		21

Y	WO, A1, 8706263 (THE GENERAL HOSPITAL CORPORATION)
	    22 October 1987,
	    see the whole document
	    --
		1-10

A	EP, A3, 0187658 (THE GENERAL HOSPITAL CORPORATION)
	    16 July 1986,
	    see the whole document
	    --
		1-17,20,
		21

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☒ Claim numbers 20-21 because they relate to subject matter not required to be searched by this Authority, namely:

   See PCT Rule 39.1(iv)

   Method for treatment of the human or animal body by means of surgery or therapy, as well as diagnostic methods.

   20,21

2. ☒ Claim numbers 1-17, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

   partially, due to the vague and inteflnite or broad wordings "capturing agent", "Bioactiv substance", "ligand", "complexing agent" etc.

3. ☐ Claim numbers............ because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the the the claims. It is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additonal fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional seach fees.

Form PCT/ISA/210 (supplemental sheet (2)) (January 1985)

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the Swedish Patent Office EDP file on   91-09-27
The Swedish Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A1- 0308208	89-03-22	AU-D-	2223988	89-03-16
		JP-A-	1221400	89-09-04
		US-A-	4859449	89-08-22
WO-A1- 8905140	89-06-15	AU-D-	2825789	89-07-05
US-A- 4863713	89-09-05	EP-A-	0251494	88-01-07
		JP-A-	63005033	88-01-11
EP-A2- 0353960	90-02-07	AU-D-	4062889	90-03-05
		JP-T-	3500419	91-01-31
		US-A-	4925648	90-05-15
		WO-A-	90/01339	90-02-22
US-A- 4932412	90-06-12	AU-D-	1087988	88-07-15
		EP-A-	0335910	89-10-11
		JP-T-	2502966	90-09-20
		WO-A-	88/04537	88-06-30
EP-A1- 0149709	85-07-31	AU-B-	568138	87-12-17
		AU-D-	3129684	85-01-31
		CA-A-	1244760	88-11-15
		JP-T-	60501906	85-11-07
		US-A-	4624846	86-11-25
		WO-A-	85/00522	85-02-14
US-A- 4624846	86-11-25	AU-B-	568138	87-12-17
		AU-D-	3129684	85-01-31
		CA-A-	1244760	88-11-15
		EP-A-B-	0149709	85-07-31
		JP-T-	60501906	85-11-07
		WO-A-	85/00522	85-02-14
WO-A1- 8706263	87-10-22	EP-A-	0249007	87-12-16
		JP-T-	2502603	90-08-23
		US-A-	4916070	90-04-10
EP-A3- 0187658	86-07-16	AU-B-	595173	90-03-29
		AU-D-	5162285	86-07-17
		JP-A-	61239889	86-10-25