

Patient-specific immunoabsorbers for the extracorporeal apheresis and methods for their preparation

Description

The invention refers to the preparation of immunoabsorbers on the basis of specific antibodies and/or antigens which, as a result of immunopathological processes proceeding, are responsible for causing or maintaining many diseases. This allows to purposefully interfere with the immunopathological regulation cycle which is responsible for the clinical consequences without impairing the whole immune system, as it is the case e.g. in the traditional therapy by medicamentous immunosuppression.

The method according to the invention allows, in addition, to enrich and prepare in a pure state immunopathologically relevant endogenic substances, thus opening up new possibilities for investigating the causes and developing therapies.

The functional basis of the immunoreaction is complex and based on the well-regulated interaction between local and system-effective cellular and humoral elements of a non-specific defence and the system of specific defence consisting of activated cells of the lymphopoietic system and the mediators and antibodies produced by them.

Depending on the type of stimulation the predominant defence activities may vary.

This augmentation of the state of defence is brought about in a natural way during an infection event or artificially, i.e. medicamentously. This is effected through the system as well as locally through the mucosae of the respiration, digestion and urogenital tracts.

By nature the organism reacts immediately to an infection. The quality and quantity of the immediate reaction depends on the type of antigen and the place of invasion. In principle, equal reactions will proceed when giving vaccines or other exogenic substances. A specific defence, measurable e.g. by the detection of specific antibodies, will become effective only after a few days. After eliminating the releasing cause the production of specific antibodies will decline and finally be stopped. After the biological degradation of antibodies only the presence of specific "memory cells" indicates which antigens the organism had to put up with in the past. Under certain circumstances, in particular, causes which mostly may no longer be stated, the organism reacts hyperactively to endogenic structures. The developing autoimmunoreaction results in a continuous destruction of the endogenic tissue the decomposition products of which, on their turn, stimulate the immune system. If this pathological regulating cycle will not be interrupted the consequences will be finally fatal, at least for the tissue concerned.

Diseases of an immunopathological genesis or participation are met frequently. Because of their chronic course and the difficulties to treat them they remarkably affect the quality of the lives of the people concerned and cause an enormous loss for national economy. One of the autoimmune diseases occurring most frequently is

rheumatoid arthritis which about 1 % of the people suffer from. The main age when this disease is manifested is about the age of 40. After 10 years approx. 50 % of the patients are incapable of earning and 10 – 20 % are most seriously disabled. The results of treatment achieved so far by immunosuppression and supporting therapies are insufficient and end frequently with stopping the therapy. After 3 years maximally only 50 % of the patients treated initially with basic therapeutical agents are under effective medication.

Owing to the frequently insufficient effectiveness and big side-effects of the traditional suppressive therapy constantly new therapeutical methods for treating autoimmune diseases are searched for. (J. Sany: Early approaches to immunotherapy of rheumatoid arthritis. EUR-J-RHEUMATOL-INFLAM: 11 (1991), 139 – 147).

These therapies are aimed at affecting humoral and cellular immune mechanisms as well as mediator systems. Here, experimental attempts are concerned which in animal experiments and clinical testing showed first successes. However, so far it was not possible to achieve a decisive breakthrough in prognosticating autoimmune diseases of patients.

For a multitude of autoimmune diseases and diseases of an immunopathological participation plasma exchange and plasma sorption were applied successfully (R. T. Baldwin, R.R. Pierce and O. H. Frazier: Guillain-Barre syndrome after heart transplantation. J-HEART-LUNGTRANSPLAN: 11 (1992), 817-819; J. Braun, J. Sieper, A. Schwarz, F. Keller, J. Heitz and H. V. Ameln: Severe lupus crisis with agranulocytosis and anuric renal failure due to a mesangial lesion (WHO IIB) – successful treatment with cyclophosphamide pulse followed by plasmapheresis (2). BR-J-RHEUMATOL: 30 (1991), 312-313; P.C. Dau: Plasmapheresis in acute multiple sclerosis: Rational and results. J-CLIN-APHERESIS: 6 (1991), 200-204; H.H. Euler, J. O. Schroeder, R. A. Zeuner and E. Treske: A randomized trail of plasmapheresis and subsequent pulse cyclophosphamide in severe lupus: Design of the LPSG trial. INT-J-ARTIF-ORGANS: 14 (1991), 639-646; D.C. Hess, K. Sethi and E. Awad: Thrombotic thrombocytopenic purpura in systemic lupus erythematosus and antiphospholipid antibodies: Effective treatment with plasma exchange and immunosuppression. J-RHEUMATOL: 19 (1991), 1474-1478; RT. Korinthenberg and M. Sauer: The Gullian-Barre syndrome in childhood. Clinical course and therapeutic measures. MONATSSCHR-KINDERHEILKD: 140 (1992), 792-798).

Plasma exchange is the oldest therapeutic method with separated plasma (membrane plasmapheresis or centrifugation) being rejected and simultaneously substituted by donor plasma or human albumin. During a treatment the simple up to double quantity of the plasma of a patient is exchanged. This method is not selective. To remove one or a few pathogenically important components the whole plasma is exchanged and substances which are essential to the patient are rejected. This has serious consequences for the patient which are tried to make good by means of various substitution therapies. In addition, the danger of transmitting pathogens such as HIV or the hepatitis pathogen arises.

In plasma sorption the plasma separated before is directed through adsorber material. Substances bonding to certain plasma components are coupled to the

adsorber material, thus removing them from the patient's plasma. If plasma sorption will be applied for removing immunologically important substances the method will be referred to as immunoadsorption. Depending on the adsorber material used this method has a varying selectivity and specificity. Various ligands and carriers were clinically used for adsorbing immunoglobulin and immune complexes from the separated plasma:

Table 1:

Examples of ligands being clinically used in extracorporeal apheresis methods

- staphylococcal protein A
- hydrophobic amino acids (tryptophan or phenylalanin)
- dextran sulfate
- aggregated IgG
- anti-human IgG
- antigens of blood groups

The following table gives a survey of autoimmune diseases treated successfully by extracorporeal immunoadsorption

Table 2:

Autoimmune diseases with successful apheresis/immunoadsorption treatment

- rapidly progressing glomerulonephritis, focal glomerulosclerosis
- systemic lupus erythematoses
- antiphospholipid syndrome
- vasculitides; e.g. periarteritis nodosa, M. Wegener
- rheumatoid arthritis
- immunological thrombocytopenic purpura
- inhibitors against coagulation factors
- hyperimmunized or ABO-incompatible prospective transplantate recipients
- polymyositis
- neurological diseases; e.g. Guillain-Barre syndrome, polyneuropathy, amyotrophic lateral sclerosis, myasthenia gravis, multiple sclerosis

Disadvantage of medicamentous therapies applied in the case of autoimmune diseases

Medicamentous immunosuppression is non-selective and non-specific. Also new immunological therapies (monoclonal or polyclonal antibodies against activation markers or receptor structures of immune cells and mediators) suppress the immune response non-selectively and/or induce, on their turn, immunity phenomena in the organism.

Disadvantages of the former apheresis and adsorption methods applied in the treatment of autoimmune diseases

The disadvantage of all apheresis/adsorption systems known so far consists, analogously to medicamentous immunosuppression, in their insufficient selectivity. This applies to the method of Balint and Hargreavans (US patent 4.681.870) already applied, immobilizing staphylococcus aureus protein A on appropriate carriers. By this method IgG and IgG complexes are removed non-specifically from the blood of patients. This refers also to the method of using carrier-coupled non-specific proteins, preferably immunoglobulins of various species, as immunoadsorbents of immune complexes described by Davis (PCT application WO 86/07152). By means of this method immune complexes, yet not the reactive individual components constantly newly formed in the case of autoimmune diseases, are eliminated.

Liberti and Pollora (US patent 4.551.435) describe a method for the elimination of substances and immune complexes from blood by adding specific antibodies of a specific concentration to patient's blood forming then immune complexes with the substance to be eliminated. They are eliminated from the blood by means of factors such as C1q, rheumatoid factors, Fc receptors and cells bearing Fc receptors immobilized on a solid carrier. The application of this method presupposes the cause to be known which is not the case in most of the cases of autoimmune diseases and that the causing antigen will have to be available in a purified condition for the production of antibodies. The immune complexes themselves are removed non-specifically, not through protein A but through biomolecules, having, due to physiological reasons, a high affinity to immunoglobulins.

It should be considered that the pathophysiologically relevant immunostructures vary in individual autoimmune diseases, even immunity phenomena of one and the same disease do so. The use of the apheresis systems so far available does not only result in an elimination of the immunopathologically relevant but also physiological immunoglobulins – which are essential to endogenic defence. The result is a general weakening of the immune system with the risk of septic complications.

The invention is aimed at making available a therapy for patients suffering from diseases which are caused by a dysregulation of the immune system or which through immunopathological processes develop into chronic forms difficult to treat. It is based on the task to develop a specific immunoadsorber for a respective patient by means of which it will be possible to remove pathogenetically important immune complexes, autoantibodies and antigens from the blood or plasma of the patients through adsorption.

This task is accomplished in conformity with claims 1 and 7, the subclaims are preferential variants.

To this end, immune complexes are removed from the plasma of the patient by means of known methods, e.g. protein A immunoadsorbents, and after having been eluted appropriately decomposed into their biologically active components. Now, the components may be coupled to an appropriate carrier material applying known

methods, e.g. gel chromatography, separately and individually or as a mixture of antibodies and antigens. With the aid of these immunoadsorbents immune complexes, antibodies and antigens important for the disease may be removed specifically from the plasma of the patient by means of plasmapheresis. These columns may be reactivated and are envisaged for multiple use. Such patient-specific immunoadsorbents may, in general, be prepared for all diseases where autoimmune complexes play a pathogenetic part.

The solution of these tasks results from the patent claims.

According to the invention patient-specific immunoadsorbents consist of antigens and/or antibodies separated from immune complexes of pathologically relevant immune factors of patients bonded to activated solid carrier materials. It contains antigens and/or antibodies separated from patients suffering from diseases which are caused or maintained by dysregulation. Autoimmune diseases or immunopathological states of reaction are, inter alia, rheumatoid arthritis, rapidly progressing glomerulonephritis, systemic lupus erythematosus, antiphosphoid syndrome, vasculitides, histoincompatible recipients of transplantates, polymyositis, neurological autoimmune diseases or immunopathological dysregulations as a result of infectious diseases. All biologically compatible substances which are able to bond covalently to their surface sufficient components of immune complexes are suited as carrier materials. Sepharose and pearl cellulose are preferably used.

The method for the preparation of patient-specific immunoadsorbents is marked by the following stages:

First of all, immune complexes are removed from the plasma of patients by means of non-selective methods, e.g. protein A immunoadsorbents, and after having been eluted appropriately they are decomposed into their biologically active components. Now, the components may be coupled to an appropriate carrier material separately or individually or as a mixture of antibodies and antigen applying known methods, e.g. gel chromatography. The decomposition of the immune complexes into their individual components in an acid or alkaline medium, preferably at pH regions of 2-5 or 10-12 which, in the case of need, after fractionation and, if necessary, after adding salts such as NaCl, MgCl₂, LiCl or urea or guanine hydrochloride which are suited to keep the reactants after reaching a certain concentration in a dissociated state are coupled to solid materials in a pH region of 2-12 applying methods, in principle, known.

With the aid of these immunoadsorbents it is possible to remove from the plasma of the patient specifically "his" immune complexes, antibodies and antigens relevant to the disease by means of extracorporeal immunoadsorption. These columns may be reactivated and are envisaged for multiple use. In general, it is possible to produce such patient-specific immunoadsorbents for all diseases where autoimmune complexes play a pathogenetic part.

In addition to being applied in therapy this method allows to isolate substances from the blood of a patient which, at least, have a share in causing the immunological dysregulation. This simplifies the investigations relating to the pathogenesis of

autoimmune diseases or diseases intensified in their course by disturbed functions. Its advantage as compared with traditional solutions is:

1. Not only immune complexes but also individual reactants so far not affected are removed.
2. A substitution of foreign immunoglobulins is no longer necessary (Transmission of diseases such as HIV is excluded, additional costs are avoided).
3. Without knowing the cause of the disease patient-specific immunoadsorbents may be prepared at low costs. Thus, specific therapeutical instruments may be provided also for such autoimmune diseases for which, owing to the low sickness rate, purposeful developments by industry are rejected for reasons of costs.
4. Antigens and/or antibodies which are responsible for causing or maintaining an autoimmune disease of an individual patient may be specifically enriched, isolated and thus provided for further investigations.

The invention will be explained in greater detail hereinafter.

Patients suffering from autoimmune diseases such as e.g. rheumatoid arthritis, lupus erythematoses or multiple sclerosis are subjected to an extracorporeal apheresis using staphylococcal protein A immunoadsorbents. Upon conclusion of an apheresis cycle the column is thoroughly washed with a buffer where detergents were added or adsorptively bonded plasma components are removed from the column by an increased ion concentration (e.g. 1-3 mol/l NaCl). The release of adsorptive plasma components is checked by electrophoresis or an immunoassay of the rinsing buffer. Thereupon, immunoglobulins, immune complexes and the dissociated immunological reactants are eluted by means of a pH gradient (e.g. citrate or acetate buffer, pH 7 - 2) or concentrated salt solutions with a varying pH (between 4 and 7). By means of electrophoresis, chromatography or other appropriate separation processes the eluted fractions are analyzed for their protein spectrum and the degree of dissociation of immune complexes.

Fractions the immune complexes of which are split up into their reactive components are used for immobilization on solid carriers. Before being coupled they may be separated upon request applying appropriate separation processes. The components of the immune complex are coupled individually or as a mixture to carrier materials activated by ONB-carbonate or H-hydroxy succinimide ethyl ester adopting known methods. After removing all components not bonded a patient-specific and regenerable immunoadsorber will be available with the aid of which only substances responsible for the immunopathological humoral dysregulation may be removed selectively from the blood of a patient.

Hereinafter, the invention will be explained in greater detail by means of examples of execution.

1st example of execution

Model experiments for the determination of the biological activity of immobilized human IgG by bonding anti-human IgG (goat) – Table 3

Human IgG was coupled to the respective carriers (sepharose 6FF and pearl cellulose) given elution conditions. C1-CO-ONB activated gel containing approx. 30 μmol of ONB-carbonate groups per ml were used for coupling human IgG.

1 ml of antiserum (5.3 mg of anti-human IgG) were diluted by 1 ml of PBS and applied to the respective carriers (flow rate: 0.1 ml/min.). The columns were washed with a few column volumes of PBS and 3 M of NaCl, pH 5.0. The elution was effected with 0.1 M of glycine HCL, 0.05 % of Tween 20, pH 2.0 (flow rate: 1.0 ml/min., 2-6 °C). The concentration of protein was determined spectrophotometrically at a wavelength of 280 nm after the eluates were neutralized with 0.5 M of K_2HPO_4 . The relative bonding capacity of antibodies per ml gel is related to the coupling of IgG given standard conditions (0.5 M of phosphate buffer, 0.05 % of Tween 20, pH 7.2).

Table 3: Determination of the biological activity of immobilized human IgG (coupled under elution conditions) by means of bonding-elution experiments with anti-human IgG- antibodies (goat) applying affinity chromatography

ONB-activated	coupling conditions	quantity of coupled human IgG mg/ml of gel	anti-human IgG bonding capacity mg antibodies/ml gel	bonding efficiency of immobilized IgG µg antibodies/mg IgG	relative carrier activity (%)
pearl cellulose	0.5 M of phosphate, pH 7.2	2.3	4.1	1800	100
pearl cellulose	0.5 M of phosphate, pH 7.2	6.2	4.1	660	100
pearl cellulose	0.1 M of citrate, pH 3.0	6.2	2.2	360	54
pearl cellulose	0.1 M of citrate, pH 3.0	3.8	4.3	1100	105
pearl cellulose	4.5 M of MgCl ₂ , pH 6.0	1.4	2.5	1790	61
sepharose 6FF	0.5 M of phosphate, pH 7.2	3.5	3.2	910	78
sepharose 6FF	0.1 M of citrate, pH 3.0	1.2	2.5	2080	61
sepharose 6FF a)	0.1 M of citrate, pH 3.0	5.0	3.3 b)	660	80
sepharose 6FF a)	0.1 M of citrate, pH 3.0	5.0	3.8 c)	760	93
sepharose 6FF	0.1 M of phosphate, pH 12.0	2.8	3.2	1140	78
sepharose 6FF	4 M of guanidine*HCl	2.0	3.7	1850	90

- a) activated with C1-CO-ONB in the presence of tertiary amines (base catalyzed); degree of activation: 20 μ mol of ONB-carbonate groups/ml of gel; coupling efficiency: 57 %
- b) 53 mg of antibodies purified by affinity chromatography in 9.2 ml of neutralized elution buffer; flow rate: 0.1 ml/min; washing buffer: PBS, 3 M of NaCl (pH 5.0); elution at a flow rate of 1 ml/min.; 63 % of the antibodies offered were eluted.
- c) 5.3 mg of antibodies purified by affinity chromatography in 9.2 ml of neutralized elution buffer; flow rate: 0.1 ml/min; washing buffer: PBS, 3 M of NaCl (pH 5.0); elution at a flow rate of 0.5 ml/min.; 70 % of the antibodies offered were eluted.

2nd example of execution

Model experiments for the determination of the bonding capacity of human IgG (antigen), immobilized by means of base-activated ONB-carbonate sepharose 6FF at a pH of 3.0 by affinity chromatography using anti-human IgG (antibodies) in excess – Table 4

Human IgG (sigma) was dissolved in the coupling buffer for coupling and filtered (0.2 μ m). This solution was added to the activated sepharose wetted by a solvent. Coupling was effected at room temperature (1 h) under careful movement. After having been blocked (1 M of ethanol amine in 0.1 M of borate, pH 8.0) for 1 h the gel was intensively washed, e.g. in a frit always with 10 times of the column volume in the following order: coupling buffer – water – 0.01 HCl – water – 24 h 0.1 borate buffer, pH 8.3 – water.

Affinity chromatography was effected at 2-6 °C with the ECONO system (Bio-Rad) using an Omnifit column 5.0 x 0.3 I.D. (350 μ l of gel). Flow rates between 0.25 and 1.0 ml/min. were chosen. The elution was measured by means of an UV-flowphotometer (280 nm). After bonding and washing with PBS the antibodies were eluted according to the flowing programme:

1. 30 min PBS
2. 60 min 3 M of NaCl pH 5
3. 30 min. PBS
4. 60 min. 0.1 M of glycine*HCl pH 2.0
5. 30 min. PBS
6. flow rate: 0.25 ml/min.

Table 4: Determination of the bonding capacity of human IgG [antigen (ag)] immobilized by means of base-activated ONB-carbonate sepharose 6FF at pH 3.0 applying affinity chromatography using an excess of anti-human IgG [antibodies (ab)]

experiment no.	immobilized ag	ab offered	ab bonding conditions		execution	bonding capacity			% ^{b)}
			mg/run	µg/ml		flow rate	PBS washing	quantity of eluted ab	
	mg/ml gel			ab/ag		ml/min.	gel volume	mg/ml gel	2ab/ag
1	5.0	6.2	1030	2.5	BA	3h RT	20	10-4	104
2	5.0	-----	-----	2.5	BAE	batch	3x1h 1x60h 400 ^{a)}	7.1	71
3	5.0	-----	-----	2.6	BAE	batch	1h 1x16h 320 ^{a)}	7.5	75
4	5.0	3.1	210	4.2	BAL	0.02	22	13.7	137
5	5.0	4.1	1050	2.3	ML	0.1	22	7.1	71
6	5.0	4.1	210	2.3	ML	0.1	86	7.7	77
7	5.0	5.7	300	3.3	ML	0.02	22	11.4	114
8	5.0	5.6	925	3.2	MC	7h 0.5	2x1h 2x16h 200 ^{a)}	9.7	97
9	5.0	7.2	210	4.1	MC	14h 0.5	5x1h 1x16h 300 ^{a)}	9.7	97
10	5.0	4.2	525 ^{a)}	2.4	MCAC	6h 0-5	2x1h 1x16h 400 ^{a)}	9.7	97

- RAT room temperature
- BA batch process
- BAE elution of the antibodies bonded in the batch process (1st experiment) after washing with PBS in the column
- Bal antibodies offered additionally to the antibodies bonded in the batch process; washing and elution through the column
- ML *micro``affinity chromatography with traditional loading by purified antibodies
- MC *micro`` affinity chromatography with loading of the column by circulation of the purified antibodies
- MCAC *micro``affinity chromatography with antiserum, diluted in PBS, by circulation
- To remove the antibodies offered in excess and/or adsorptively bonded proteins it was washed with PBS. Washing was interrupted by a few hourly intervals (or 16 h) before the elution programme was started.
 - The quantity of the anti-human IgG (antibodies) was spectrophotometrically determined at λ_{280} ($E^{0.1\%} = 1.38$) after neutralization with 0.5 M of K_2HPO_4 . A bonding capacity of 100 % is assumed if each immobilized IgG molecule will bond 2 molecules of anti-IgG
 - antiserum, diluted 1:8 in PBS

3rd example of execution

- Elution of anti-HSA (rabbits) by HSA from antibody-antigen complexes applying HSA-coated microtitration plates for the determination of optimum elution conditions for affinity chromatography (Table 5).

The 96 well microtitration plates were coated with HSA. Each well was incubated with 0.1 μ g of anti-HSA. Anti-rabbit IgG conjugated with alkaline phosphatase (substrate: 4-nitrophenyl phosphate, λ_{405} nm) served as detection system (ELISA). 200 μ l of the respective elution buffer were pipetted into the wells. The elution was effected at room temperature in the course of an hour with the microtitration plate being moved constantly. After the wells had been washed thoroughly anti-HSA was detected. For the evaluation the average value of the measurements of always 8 wells was formed (% CV = 4.4). The percentage of anti-HSA antibodies eluted with PBS was fixed with 0 %.

Table 5:

Elution buffer for dissolving the antibody-antigen complexes
eluted anti-HSA antibodies (%)

PBS, pH 7.3	0
PBS + 1 % of SDS, pH 7.3	51
0.10 M of citrate, pH 2.5*	100
0.10 M of citrate, pH 3.0*	73
0.10 M of citrate, pH 3.5*	27
0.10 M of citrate, pH 4.0*	8
0.10 M of citrate, pH 2.5*	100
0.1 M of citrate/phosphate, pH 7.3	0
0.1 M of citrate/phosphate, pH 6.0	0
0.1 M of citrate/phosphate, pH 5.0	0
0.1 M of citrate/phosphate, pH 4.0	2
3.00 M of KSCN, pH 7.3	41
3.00 M of NaCl, pH 5.0*	0
3.00 M of guadinine*HCl, pH 7.3	66
4.00 M of guadinine*HCl, pH 7.3	90
6.00 M of urea, pH 7.3	7
0.10 M of borate, pH 11.0	35
0.10 M of phosphate, pH 11.5	49
0.10 M of phosphate, pH 12	91

- with 0.015 % of Tween 20

- a) Immobilization of anti-HSA-HSA on base catalyzed, activated ONB-carbonate sepharose 6FF in coupling media used as elution media for immunoaffinity chromatography (Table 6)

The coupling media where protein had been added and which were filtered (0.45 μ m) were added to the wet activated ONB-carbonate sepharose 6FF. Coupling was effected for one hour at room temperature under slight movement. Thereupon, blocking was effected for one hour at room temperature with ethanol amine in borate buffer (pH 8.1). The ONB-carbonate groups were determined spectrophotometrically (λ_{max} : approx. 267 nm).

0.5 M of phosphate buffer, pH 7.3, served to determine the reference value for a maximum immobilization.

Washing was carried out as in the 2nd example.

Anti-HSA antibodies were obtained by a preliminary experiment by means of affinity chromatography (0.05 – 0.1 M of citrate, pH 2.0), neutralized with 0.5 M of K_2HPO_4 , stored at – 20 °C and after thawing set to a pH of 3.0 or 4.0 by diluted HCl for coupling.

HSA was dissolved in PBS, set to the respective pH by diluted HCl and added to the antibody solution to couple the antigen-antibody mixture.

The protein was determined spectrophotometrically $OD_{280\text{ nm}}$ (antibodies $E^{0.1\%} = 1.38$ and HSA $E^{0.1\%} = 1.67$) in the coupling buffer. After treating the gels with 1 N NaOH in the supernatant liquid according to Lowry the immobilized proteins were determined.

The result of coupling (%) is the relative quantity of immobilized protein related to the protein quantity offered.

Table 6: Immobilization of anti-HSA-HSA on base-catalyzed, activated ONB-carbonate sepharose 6FF in coupling media used as elution media for immunoaffinity chromatography

μmol ONB-carbonate groups per ml gel	"coupling medium	pH	mg/ml gel offered	protein quantity mg/ml solution	immobil. protein mg/ml gel	result of coupling %
19.8	0.5 M of phosphate	7.3	1.6 A	0.3	1.1	70
31.6	0.5 M of phosphate	7.3	3.9 HSA	3.9	1.7	43
5.1	0.1 M of phosphate	7.3	1.6 A	0.3	0.6	34
22.3	elution buffer	3.0	3.2 A	0.6	2.3	70
20.3	elution buffer	3.0	5.0 D	1.6	3.4	67
10.8	elution buffer	3.0	3.8 B	0.5	2.3	60
19.8	elution buffer	4.0	3.8 B	0.7	1.2	32
22.3	elution buffer	3.0	5.7 C	0.9	2.9	50
19.8	4 M of guanidine *HCl	7.3	11.3 B	11.3	4.9	43
22.3	0,1 M of phosphate	12.0	4.8 B	2.0	1.3	28

- A anti-HSA
 B anti-HSA/HSA 1 : 1
 C anti-HSA/HSA 1 : 2
 D anti-human IgG/human IgG 1 : 1

4th example of execution

Detection of the biological activity (bondability) of antigens and antibodies by means of the anti-HSA/HSA model after immobilization given splitting conditions for immune complexes (Table 7)

Given elution conditions (pH 3.0; pH 4.0; 4 M of guanidine*HCl) as described in the 1st and 3rd examples of execution base catalyzed, activated ONB-carbonate sepharose 6FF was coupled to anti-HSA/HSA. After washing of the column HSA or anti-HSA were offered in the bonding buffer. After repeated washing of the column with PBS the elution (pH 2.0) and photometric determination of the protein concentration were effected.

By these model experiments it was detected that the antigen (HSA) and the antibodies (anti-HSA) from immune complexes (anti-HSA/HSA) immobilized on a carrier under splitting conditions maintain their bondability. The immobilized HSA from immune complexes has always bonded anti-HSA which after repeated elution in ELISA was characterized by a high reactivity to HSA (results were not represented). Assuming that 1 mol of HSA bonds one mol of anti-HSA 0.9 mg of HSA/ml of gel from immune complexes bonding 2.1 mg of anti-HSA were immobilized (3rd experiment). Similar results could be reproduced by the experiments 5, 7, 13 and 16. Anti-HSA (rabbit IgG) immobilized from immune complexes is also efficiently immobilized in this model given standard coupling conditions - the success may be proved by anti-rabbit IgG – yet, coupling is here, obviously, effected in a molecule region resulting in steric hindrances for bonding HSA. The antibodies themselves maintain their biological activity.

Table 7: Detection of the biological activity (bondability) of antigens and antibodies at the model of anti-HSA/HSA after their immobilization on base-catalyzed, activated ONB-carbonate sepharose 6FF under decomposition conditions for immune complexes

immobilization conditions ^{a)}	protein offered /ml gel	concentration µg/ml	bonding conditions	PBS washing	bonding capacity µg/ml gel
1. 10.8 µmol anti-HSA/HSA 1:1, pH 3 2.3 mg/ml	3.0 mg HSA	1055	0.3 ml/min, 43 min. circulation	0.028 ml/min, 43 min.	100-200
2. 10.8 µmol anti-HSA/HSA 1:1, pH 3 2.3 mg/ml 10.8 µmol	13.4 mg HSA	937	0.5 ml/min, 120 min. circulation	0.5 ml/min, 60 min.	100-200
3. anti-HSA/HSA 1:1, pH 3 2.3 ml/ml 19.8 µmol	3.7 mg anti-HSA	164	0.5 ml/min, 135 min circulation	0.5 ml/min, 60 min	2100 (= 0.9 mg HSA)
4. anti-HSA, pH 7 1.1 mg/ml 19.8 µmol	6.1 mg HSA	531	0.5 ml/min, 120 min. circulation	0.5 ml/min, 60 min.	100-200
5. anti-HSA/HSA 1:1, pH 4 1.2 mg/ml 19.8 µmol	4.2 mg anti-HSA	368	0.5 ml/min, 180 min. circulation	0.5 ml/min, 60 min	1211 (= 0.5 mg HSA)
6. anti-HSA/HSA 1:1, 4 M of guan. 4.9 mg/ml	8.3 mg HSA	578	0.5 ml/min, 90 min. circulation	0.5 ml/min, 60 min.	traces

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7.	19.8 µmol anti-HSA/HSA 1:1, 4 M of guan. 4.9 mg/ml 19.8 µmol	7.3 mg anti-HSA	470	0.5 ml/min, 180 min. circulation	0.5 ml/min, 60 min.	4714 (= 2.1 mg HSA)
8.	anti-HSA/HSA 1:1, 4 M of guan. 4.9 mg/ml 10.8 µmol	5.1 mg anti-rabbit IgG 360	360	0.5 ml/min, 120 min. circulation	0.5 ml/min, 60 min.	1977 (36 %)
9.	anti-HSA/HSA 1:1, pH 3 2.3 mg/ml 19.8 µmol	5.1 mg anti-rabbit IgG 360	360	0.5 ml/min, 120 min. circulation	0.5 ml/min, 60 min.	1480 (54 %)
10.	anti-HSA, pH 7 1.1 mg/ml 22. ml/mol	5.1 mg anti-rabbit IgG 360	360	0.5 ml/min, 120 min. circulation	0.5 ml/min, 60 min.	2246 (100 %)
11.	anti-HSA, pH 3 2.3 mg/ml 22.3 µmol	5.1 mg anti-rabbit IgG 360	360	0.5 ml/min, 120 min. circulation	0.5 ml/min, 60 min.	2246 (48 %)
12.	anti-HSA, pH 3 2.3 mg/ml 22.3 µmol	13.8 mg HSA	926	0.5 ml/min, 120 min. circulation	0.5 ml/min, 60 min.	100-200
13.	anti-HSA/HSA, 1:2, pH 3 2.9 mg/ml 22.3 µmol	6.9 mg anti-HSA	477	0.5 ml/min, 120 min. circulation	0.5 ml/min, 60 min.	3586 (= 1.6 mg HSA)
14.	anti-HSA/HSA, 1:2, pH 3 2.9 mg/ml	11.2 mg HSA	784	0.5 ml/min, 120 min. circulation	0.5 ml/min, 60 min.	100-200

15.	22.3 μ mol anti-HSA/HSA, 1:1, pH12 1.3 mg/ml	6.8 mg HSA	600	0.5 ml/min, 70 min. circulation	0.5 ml/min, 60 min.	100-200
16.	22.3 μ mol anti-HSA/HSA, 1:1, pH12 1.3 mg/ml	4.4 mg HSA	424	0.5 ml/min, 120 min. circulation	0.5 ml/min, 90 min.	1579 (=0.7 mg HSA)

a) indication of μ mol: active groups per M of gel

5th example of execution

Affinity chromatography of the plasma of a patient suffering from lupus erythematoses (at the same time standard method)

The plasma of a patient suffering from lupus erythematoses was available. A protein A column (PHARMACIA) was applied for obtaining the total γ -globulines. The immobilization of the antibodies was effected through ONB-carbonate activated sepharose 6FF (20 μ mol/ml).

Buffer solutions for immunoadsorption

buffer PA, pH 7.0	1000 ml	The pH is set by HCl or NaOH.
trisodium citrate	3.30 g	
sodium acetate x 3 H ₂ O	5.45 g	
sodium chloride	4.90 g	
disodium hydrogenphosphate	2.91 g	
potassium dihydrogenphosphate	0.26 g	
eluant PA, pH 2.2	1000 ml	The pH is set by HCl or NaOH.
citric acid x H ₂ O	6.12 g	
sodium chloride	9.00 g	
washing buffer 3 M of NaCl pH 7.0	1000 ml	The pH is set by HCl or NaOH.
trisodium citrate	3.30 g	
sodium acetate x 3 H ₂ O	5.45 g	
sodium chloride	175.00 g	
disodium hydrogenphosphate	2.91 g	
potassium dihydrogenphosphate	0.26 g	
Tween 20	0.50 g	

citrate buffer 0.1 M, pH 2.2
citric acid

250 ml
5.25 g

The pH is set by HCl or NaOH.

Methods:

A protein A-coupled column (5 ml of gel, Pharmacia) was equilibrated with buffer PA. 20 ml of high-speed centrifugated, fresh plasma were mixed 1:2 with buffer PA and applied. For the chromatography we used an Econo system (BIORAD). After leaving the column the plasma was anew applied to achieve a complete adsorption. Intensive washing with 5 column volumes of PA was required to remove the material not adsorbed. Non-specifically bonded proteins were removed by a washing buffer containing 3 M of NaCl. The immunoglobulins and proteins eluted from the immune complexes as a sharp peak by 0.1 M of citrate buffer, 0.05 % of TWEEN 20, pH 2.2. The volume of the eluate totalled 6.5 ml. The protein concentration was determined by means of UV-adsorption at λ_{280} nm with 17.6 mg/ml. Immediately after elution IgG and proteins available separately were coupled to the ONB-carbonate activated sepharose. To this end, 6 ml of the gel prepared according to the manufacturer's instruction and sucked off were added to the eluate and shaken for 1 h. Owing to the buffer effect of the proteins dissolved there the eluate has a PH between 3 and 4. Free bonds had to be saturated by 1 M of ethanol amine in 0.1 M of borate buffer, pH 8.0. By comparing the protein concentrations in the combined washing solutions with the protein A eluate we stated a bonding efficiency of 57 %. After thorough washing the gel is available as carrier for affinity chromatographic experiments.

For this purpose, 40 ml of patient's plasma were centrifugated, diluted 1: 2 by PA and put through the column 2 times. Non-bonded or non-specifically bonded material were removed by subsequent washing with always 10 column volumes of PA, washing buffer and PA. The specifically bonded protein was eluted by 0.1 M of citrate buffer, pH 2.2 (Fig. 1).

The plasma proteins and the chromatographically obtained fractions were analyzed according to standard methods in the course of SDS polyacrylamide gel electrophoresis (SDS-PAGE) (Miniprotean II, BioRad). The gels were gradient gels of 10 – 25 % monomer concentration. Colouring was carried out by coomassie brilliant blue R-250 (Figs. 2 and 3).

From Fig 2 there can be seen that still further proteins are contained in the protein A eluate in addition to antibodies. After their immobilization they are in a position to specifically bond the respective reactants from the patient's plasma. In the elution peak 2 (0.1 M of citrate buffer, pH 2.2) a few proteins were identified in PAGE after affinity chromatography. In addition to immunoglobulins and a few higher molecular proteins 3 proteins, according to PAGE of the protein A eluate detectable only as scarcely visible bands, were decisively enriched (Fig. 3). They show a relative mol mass of about 40 kD.

6th example of execution

Affinity chromatography of the plasma of a patient suffering from multiple sclerosis

The treatment of the plasma, elution of protein A and coupling of the eluate proteins were effected analogously to the 5th example of execution. After washing adsorptively bonded proteins from the immunoabsorber (peak 1, Fig. 4) specifically bonded protein is dissolved from the matrix by means of the elution buffer which, according to PAGE (Fig. 5), proved to be a protein mixture containing mainly immunoglobulins.

7th example of execution

Affinity chromatography of the plasma of a patient suffering from rheumatoid arthritis

The treatment of the plasma, elution of protein A and coupling of the eluate proteins were effected analogously to the 5th example of execution. After washing adsorptively bonded proteins from the immunoabsorber (peak 1, Fig. 6) specifically bonded protein is dissolved from the matrix by means of the elution buffer which, according to PAGE (Fig. 7), proved to be a protein mixture containing mainly immunoglobulins.

Legend for the Figures 1 to 7

Fig. 1:

Elution profile of the plasma of a patient after adsorption to sepharose 6FF coupled to homologous antibodies and antigens according to the standard method

The marks give the exchange of buffer during washing and elution (loading of the column is not indicated):

no. 1 – washing buffer, no. 2 – 0.1 M of citrate buffer pH 2.2

Fig. 2:

PAGE of the eluate from the protein A column before and after coupling to the ONB-carbonate activated sepharose

no. 1 / 2 = 5 or 10 µl of material before coupling, no. 3 = 10 kD conductor, no. 4 = γ-globulin standard, nos. 5-8 as 1-4, yet after coupling (i.e. not bonded material)

Fig. 3:

PAGE of eluate which was obtained from the immunoabsorbent column at pH 2.2 after passing the homologous plasma

nos. 1-7/9-15 = peak fractions, nos. 8/17 = 10 kD protein marker, no. 16 = rinsing

Fig. 4:

Elution profile of the plasma of a patient after adsorption to sepharose 6FF coupled to the homologous antibodies and antigens according to standard methods

The marks give the exchange of buffer during washing and elution (loading of the column is not indicated):

no. 1 – washing buffer, no. 2 – 0.1 M of citrate buffer pH 2.2

Fig. 5:

PAGE of eluate which was obtained from the immunoabsorbent column at pH 2.2 after passing the homologous plasma

no. 1 = 10 kD standards, no. 2 = γ-globulin standard, no. 3 = peak fraction (peak 2, Fig. 4), no. 4 = peak fraction after evaporation through amicon centrifree®

Fig. 6

Elution profile of the plasma of a patient after adsorption to sepharose 6FF coupled to the homologous antibodies and antigens according to standard methods

The marks give the exchange of buffer during washing and elution (loading of the column is not indicated):

no. 1 – washing buffer, no. 2 – 0.1 M of citrate buffer pH 6.0/Tween, no. 3 – 0.1 M of citrate buffer, pH 2.2

Fig. 7:

PAGE of eluate which was obtained from the immunoabsorbent column at pH 2.2 after passing the homologous plasma

nos. 1-4 = proteins from the washing buffer, nos. 5/6 = peak fractions (peak 3, Fig. 6), no. 7 = 10 kD standards, no. 8 = γ -globulin standard

**Patent claims**

1. Patient-specific immunoadsorbents consisting of antigens and/or antibodies separated from immune complexes of pathologically relevant immune factors of patients, bonded to activated solid carrier materials
2. Patient-specific immunoadsorbents according to claim 1 containing antigens and/or antibodies separated from patients suffering from diseases which are caused or maintained by a dysregulation of the immune system
3. Patient-specific immunoadsorbents according to claims 1 and 2 containing antigens and/or antibodies separated from patients suffering from autoimmune diseases or immunopathological states of reactions such as rheumatoid arthritis, rapidly progressing glomerulonephritis, systemic lupus erythematoses, multiple sclerosis, antiphosphoid syndrome, vasculitides, histoincompatible recipients of transplantate, polymyositis, neurological autoimmune diseases or immunopathological dysregulations as a result of infectious diseases
4. Patient-specific immunoadsorbents according to claims 1 -3 containing antigens and/or antibodies separated from patients suffering from rheumatoid arthritis, lupus erythematoses or multiple sclerosis
5. Patient-specific immunoadsorbents according to claims 1 - 4 bonded to biocompatible substances which may bond covalently on their surface sufficient immune complex components.
6. Patient-specific immunoadsorbents according to claims 1 - 5 bonded to sepharose or pearl cellulose.
7. Method for the production of patient-specific immunoadsorbents according to claims 1-6 wherein immune complexes are isolated from the blood or other body fluids such as e.g. liquor cerebrospinalis of patients applying known methods such as plasmapheresis through protein A immunoadsorbents, immune complexes are purified for use in clinics in extracorporeal blood purification methods and are split into their active components antibodies and antigens and these components are covalently coupled to solid carrier materials.

THIS SHEET HAS BEEN
REPLACED



8. Methods according to claim 7 wherein immune complexes are decomposed into their individual components in an acid or alkaline medium, preferably at pH regions of 2-5 or 10 – 12 and/or with salts such as NaCl, MgCl₂, LiCl or urea or guanine hydrochloride being added and, in the case of need, after fractionation and, in the case of need, after adding salts such as NaCl, MgCl₂, LiCl or urea or guanine hydrochloride which are suited for maintaining the reactants in a dissociated state from a certain concentration on, in a pH region between 2 and 12 are coupled to solid materials by means of, in general, known methods
9. Use of patient-specific immunoadsorbents according to claims 1-6 for the extracorporeal aphoresis
10. Use of patient-specific immunoadsorbents according to claims 1-6 for obtaining and further treating immunopathologically relevant endogenic substances

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REPLACED



1/3

Abb. 1:

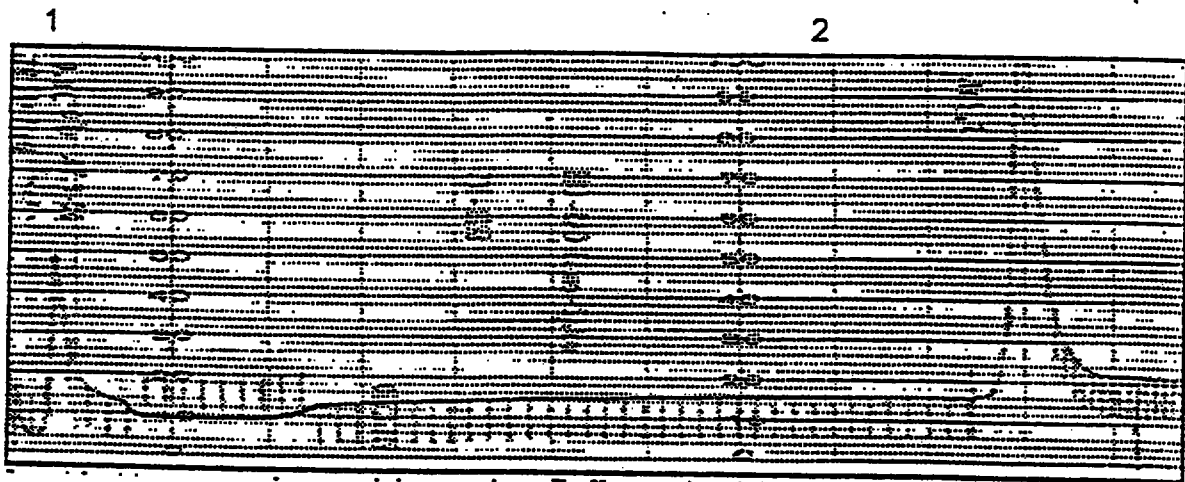


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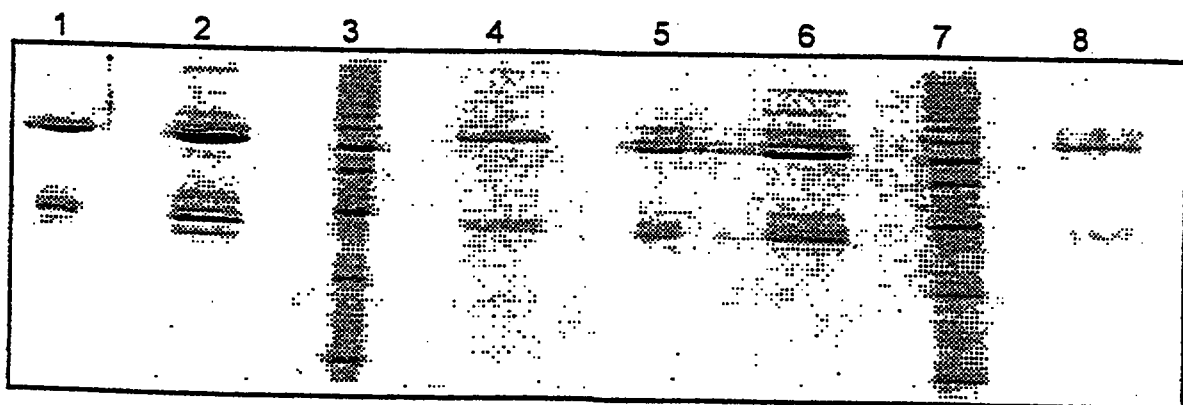
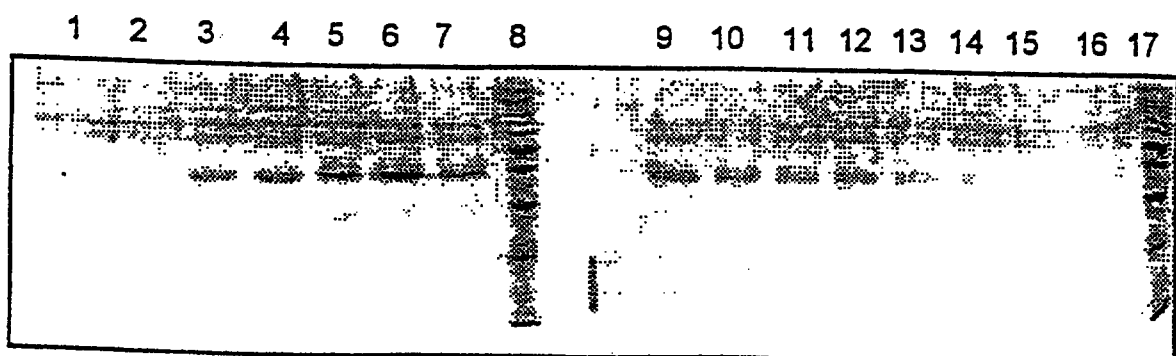


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2/3

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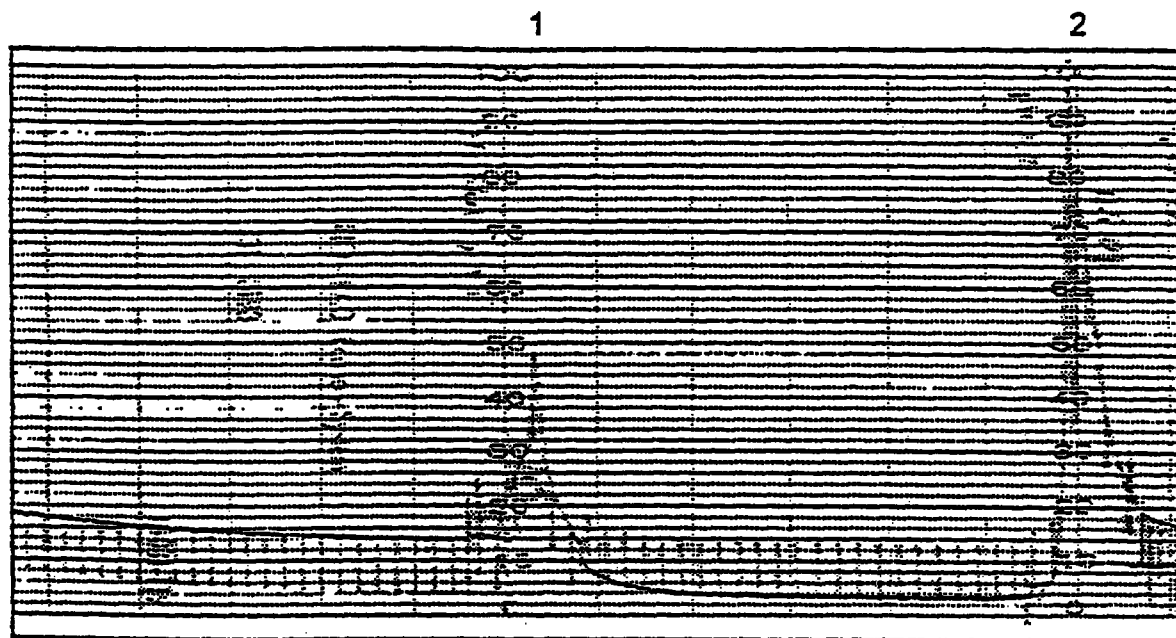
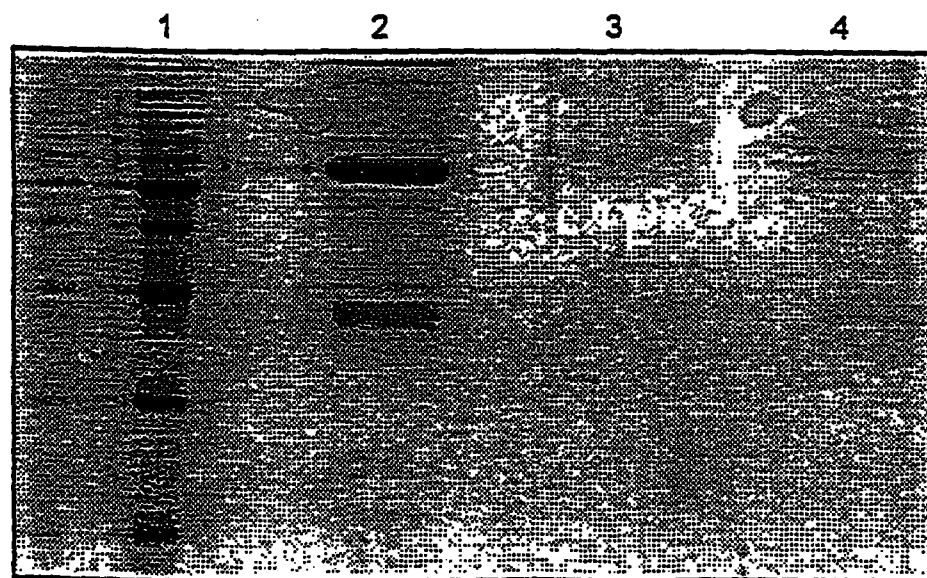


Abb. 5:



3/3

Abb. 6:

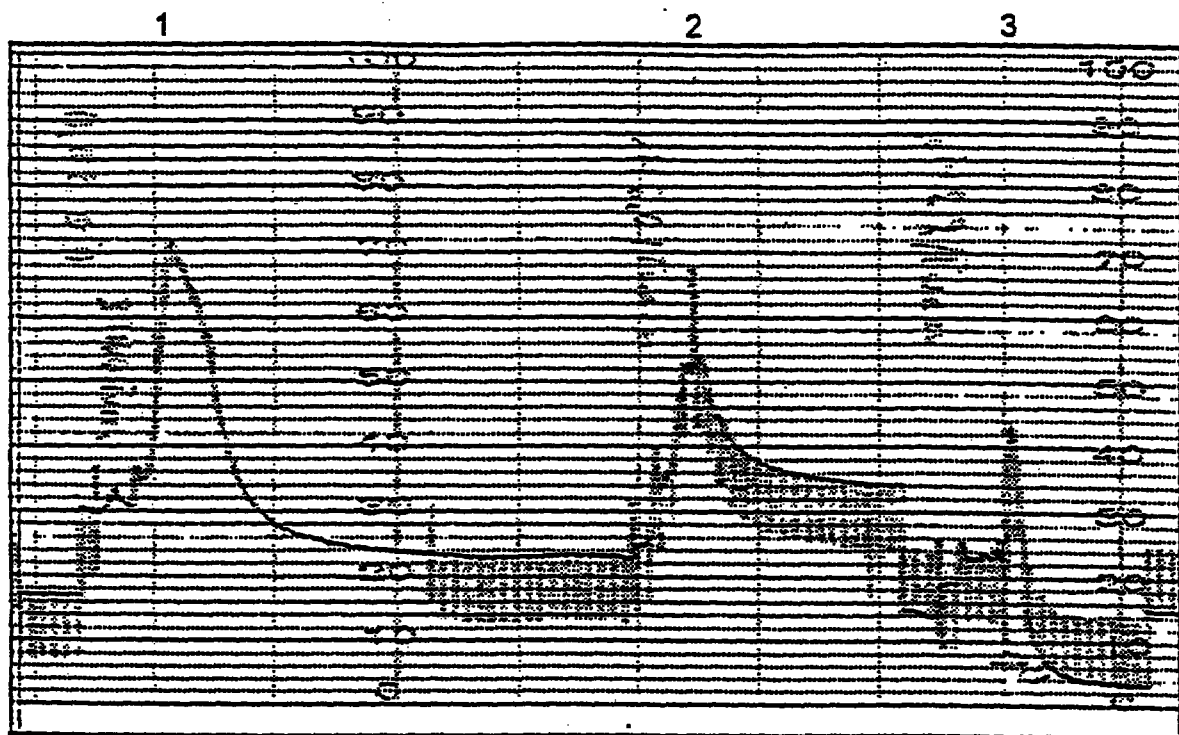


Abb. 7:

