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Tandem immunoaffinity and reversed-phase high-performance liquid chromatography for the identification of the specific binding sites of a hapten on a proteic carrier

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ABSTRACT

Immunoaffinity (IA) and reversed-phase (RP) high-performance liquid chromatography were combined for the identification of the specific binding sites of benzylpenicilloyl (BPO) groups on human serum albumin (HSA). Tryptic hydrolysates of BPO–HSA were loaded on the IA column. BPO–peptides were desorbed and concentrated directly on the RP column, coupled via a switching valve, then separated by using gradient elution and identified by the amino acid sequences. This single-step procedure permitted more than 95% recovery of the BPO–peptides present in minute amounts, with good specificity.

INTRODUCTION

Like most drugs, penicillin is not immunogenic by itself. Allergic accidents occurring after penicillin therapy or consumption of food from penicillin-treated animals are mainly due to benzylpenicilloyl (BPO)–protein conjugates. These allergenic compounds result from the cleavage of the β-lactam ring of penicillin G and formation of covalent bond between the carbonyl of BPO and ε-amino groups of proteins. In man or animals treated with penicillin, the main proteic carrier for BPO is the serum albumin. *In vitro*, under physiological conditions of pH and temperature, human serum albumin (HSA) can also be penicilloylated to form HSA–BPO conjugates similar to those occurring *in vivo*. The mechanism of protein penicilloylation, based on a nucleophilic attack of penicillin by NH_2 groups of albumin, needed to be confirmed as previous observations suggested that among the various (*ca.* 60) ε-amino groups of lysine present in albumin, only a few of them were available for penicillin aminolysis [1].

The study of the specific binding sites of a ligand (such as a hapten) covalently bound on a proteic carrier needs first the preparation and the chromatographic separation and purification of peptides from the original conjugate and second hapten detection on each peptide using a specific method. This procedure was used by Walker

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[2] to identify the binding site of acetylsalicylic acid on human serum albumin. We have localized BPO groups on a fragment of albumin from a penicillin-treated patient by the same method, which needs numerous steps and is time consuming [3].

Several workers have used a two-step approach; the first is affinity chromatography and the second reversed-phase high-performance liquid chromatography (RP-HPLC) to separate the peptides retained on the affinity column. This approach was used by Iberg and Flückiger [4] to identify the glycosylated sites on albumin from a diabetic patient and by Lafaye and Lapresle [5] to search for BPO-binding sites on HSA from penicillin-treated patients. However, the separation of peptides on a low-pressure affinity column takes several hours and often needs large amounts of material (100–300 mg of proteins).

Tandem immunoaffinity and RP-HPLC have already been used for various studies such as the determination of recombinant leucocyte interferon α-2 in complex mixtures [6] and to discriminate between similar structural forms of proteins [7]. We have tried to use this method to isolate specifically penicilloylated peptides from hydrolysates of BPO–HSA conjugates obtained from penicillin-treated patients. Only very small amounts of such samples were available. We compared this binding with that occurring on a conjugate prepared *in vitro*.

EXPERIMENTAL

Reagents and penicilloylated albumins

For HPLC, all the chemicals used were of HPLC grade. For amino acid analysis and for recurring Edman degradation, the chemicals used were of Sequanal grade (Pierce). Other reagents were of analytical-reagent grade.

Penicilloylated albumins prepared from sera from three penicillin-treated patients [1,3] were obtained from the Institut Pasteur (Paris, France). A BPO–HSA conjugate was prepared *in vitro* as described [3].

Approximately two BPO groups were covalently fixed per mole of HSA in the case of the *in vitro* conjugate. Concerning the penicilloylated albumins obtained from the patients, 2.8–3 BPO groups were determined per mole of HSA. However, it should be noted that in each instance, these penicilloylation rates are mean values corresponding to the average number of BPO groups measured on the whole population of penicilloylated albumin molecules. It does not mean that all the molecules have fixed two (or three) BPO groups; some might have fixed more BPO groups whereas others might have fixed only one group.

Digestion of penicilloylated albumins

Cyanogen bromide digestion of penicilloylated albumins and HPLC separation of fragments

Cyanogen bromide (CNBr) cleavage was carried out on 2 mg of BPO–albumin and separation of fragments $A_{299\text{-}585}$, $B_{1\text{-}123}$ and $C_{124\text{-}298}$ was performed by RP-HPLC as described previously [8].

Reduction and carboxymethylation of the CNBr fragment C

Denaturation, disulphide cleavage and alkylation were performed as described by Swenson *et al.* [9]. A 10-nmol sample of fragment C was dissolved in 1 *M* Tris–HCl buffer (pH 8.0) containing 6 *M* urea and a 10-fold molar excess of dithiothreitol over the SH concentration and kept overnight at 40°C under a nitrogen atmosphere.

Iodoacetic acid in 1 *M* NaOH (1.3-fold molar excess over total thiols) was added and the mixture was maintained in the dark for 4 h, before the reaction was stopped by addition of 5 μl of β-mercaptoethanol. Desalting of the mixture was performed by RP-HPLC: 100 μl were injected onto an Aquapore RP-300 (7 μm) column (30 × 4.6 mm I.D.) (Brownlee), equilibrated in a mixture of 50% solvent A [0.05% aqueous solution of trifluoroacetic acid (TFA)] and 50% solvent B [acetonitrile–2-propanol–0.05% TFA in water (2:1:2, v/v/v)]. Elution of fragment C was achieved with a 30-min linear gradient from 50 to 100% of solvent B at a flow-rate of 1.0 ml/min. Runs were performed at room temperature and peptides were detected at 215 nm. The reduced C-containing fractions were pooled and the solvent was removed using a Speed-Vac evaporator.

Tryptic digestion

Tryptic digestion was performed on reduced fragment C in 0.1 *M* phosphate buffer (pH 7.8) overnight at room temperature using L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK)–trypsin (Sigma) (molar ratio enzyme/substrate, E/S = 1/50). The enzymatic reaction was stopped by heating at 100°C for 10 min.

Isolation and separation of the penicilloylated fragments from tryptic digest

Preparation of immunoaffinity column (IAC)

Purification of antipenicilloyl antibodies. Rabbit anti-BPO antisera were obtained as described previously [10]. An HSA–BPO conjugate (5 mol BPO/mol HSA) was prepared at alkaline pH and in the presence of a 10-fold molar excess of penicillin.

An Ultraffinity-EP column (50 × 46 mm I.D.) (Beckman) was derivatized by recycling a solution of HSA–BPO conjugate (200 mg) in 20 ml of 1 *M* potassium phosphate buffer (pH 7.0) at 0.2 ml/min for 18 h. The derivatized column was then washed with 0.1 *M* potassium phosphate (pH 7.0) at 1.0 ml/min for 1 h and converted to the loading buffer [0.02 *M* phosphate–0.2 *M* NaCl (pH 7.0)].

Rabbit anti-BPO antisera were dialysed with the loading buffer and 5–10 ml were loaded onto the column at 1.0 ml/min. The column was washed with 10 ml of loading buffer at the same flow-rate. The anti-BPO antibodies were eluted with 7 ml of 0.01 *M* HCl (pH 2) at the same flow-rate. The column was then re-equilibrated with loading buffer.

Column derivatization with antipenicilloyl antibodies. A 13-mg sample of antipenicilloyl antibodies purified as described before was dissolved in 18 ml of 1 *M* phosphate buffer (pH 7.0) and recycled through an Ultraffinity-EP column (50 × 4.6 mm I.D.) overnight at 0.2 ml/min. An 8-mg sample of antibodies was then immobilized. The column was washed with 1 *M* phosphate buffer (pH 7.0) at 1.0 ml/min for 1 h and stored in the same buffer until needed. It was equilibrated with 10 m*M* phosphate buffer (pH 7) containing 8 g/l NaCl [phosphate-buffered saline (PBS)] before use.

Apparatus

The immunoaffinity column was connected to a reversed-phase column (Nucleosil $5C_{18}$, 250 × 46 mm I.D.) (Société Française Chromato Colonne, Neuilly-Plaisance, France) via a six-way valve (Rheodyne, Cotati, CA, U.S.A.) as described by Rybacek *et al.* [6]. The switching valve contained the affinity column in place of a sample loop. The position of the switching valve determined whether the immunoaffinity and analytical columns would be used in series or separately.

Production and separation of the penicilloylated fragments

The tryptic digests of peptides C were loaded onto the affinity column in PBS at a flow-rate of 1.0 ml/min. The column was then washed with 10–15 ml of PBS at the same flow-rate. Non-retained peptides were collected to check the absence of BPO, then the valve was switched to place both columns in-line. The bound antigens were desorbed from the immunoaffinity column into the analytical column with 0.1% TFA solution at a flow-rate of 1.0 ml/min. After 10 min, the valve was switched, placing the immunoaffinity column off-line to permit the analysis of the antigens by gradient elution on the analytical column. The reversed-phase chromatographic separation was performed with a 30-min linear gradient from 0 to 100% of acetonitrile–isopropanol–0.1% TFA in water (2:1:2, v/v/v) at a flow-rate of 1.0 ml/min. All runs were performed at room temperature and peptides were detected at 215 nm. Fractions were collected every 0.25 min and analysed for BPO concentration. Simultaneously, the affinity column was equilibrated with loading buffer.

RP-HPLC at pH 7.0 was performed on the same column equilibrated with 0.01 *M* phosphate buffer (pH 7.0). Elution was achieved with a 30-min linear gradient from 0 to 100% of acetonitrile–0.01 *M* phosphate buffer (pH 7) (60:40, v/v) at a flow-rate of 1.0 ml/min.

Detection of penicilloyl groups

BPO detection was performed using an enzyme immunoassay (EIA) directly derived from the radioimmunoassay as described [10].

Identification of peptides and location of BPO-binding sites

Sequencing was achieved using a Model 477 Sequanator connected to a Model 120 A PTH HPLC analyzer (Applied Biosystems).

A recurring Edman degradation using Tarr's procedure [11] was performed on each peptide and the EIA for BPO detection was realized at each cycle on the phenylthiohydantoin (PTH) derivative

RESULTS

Isolation and separation of penicilloylated peptides

The isolation and separation of penicilloylated peptides from C tryptic digests were achieved using the dual-column chromatographic system described above. Fig. 1 shows the RP-HPLC patterns of (a) the entire tryptic hydrolysate generated on the

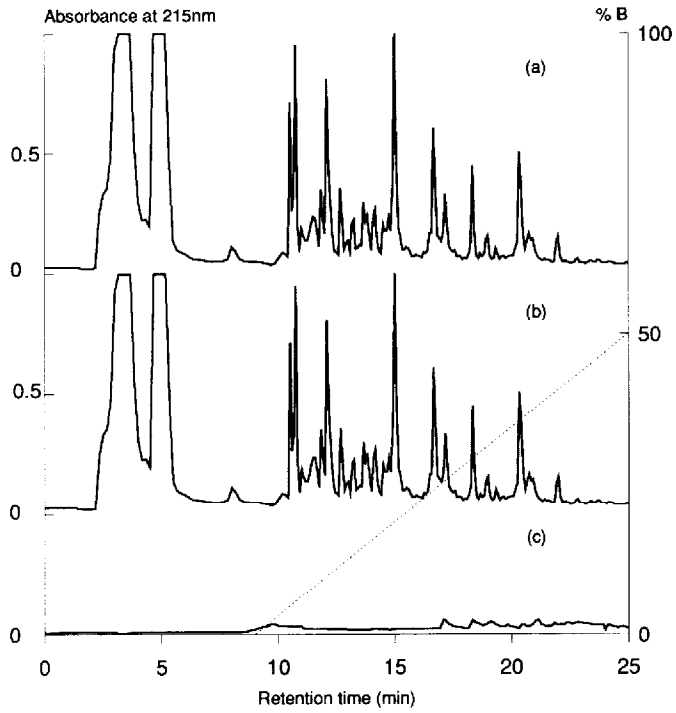

Fig. 1. RP-HPLC profile of (a) the entire tryptic hydrolysate of HSA fragment C, (b) the non-retained fraction and (c) the retained fraction on the immunoaffinity column. See text for the chromatographic conditions. The dotted line indicates the gradient of solvent B.

reversed-phase column alone, (b) the non-retained fraction on the IAC and (c) the retained fraction. Almost all the peptides of the hydrolysate did not bind to the IAC, as the chromatograms of the entire hydrolysate and of the non-retained fraction were nearly identical. The immunoenzymatic assay applied to the non-retained fraction showed no BPO present in this fraction whereas the loaded sample contained 1.2 μg of BPO.

The chromatogram of the retained peptides and the BPO assay in the collected fractions (Fig. 2) show two major BPO-containing peaks, *i.e.*, Pep 17 and Pep 21 at retention times of 17.1 and 21.1 min, respectively. The sum of the BPO in all the collected fractions was 1.12 μg, which represents about 95% of the loaded amount.

Identification of peptides and location of BPO-binding sites

Each of the BPO–peptides was analysed for its amino acid sequence. In order to confirm the BPO-binding site, BPO determinations were performed again at each cycle of the Edman degradation. The amino acid sequencing permitted the following peptides to be identified.

Pep 17 consists of a mixture of two peptides corresponding to the sequences 187–195 and 191–197 of HSA. They can be separated by RP-HPLC at pH 7.0 into two

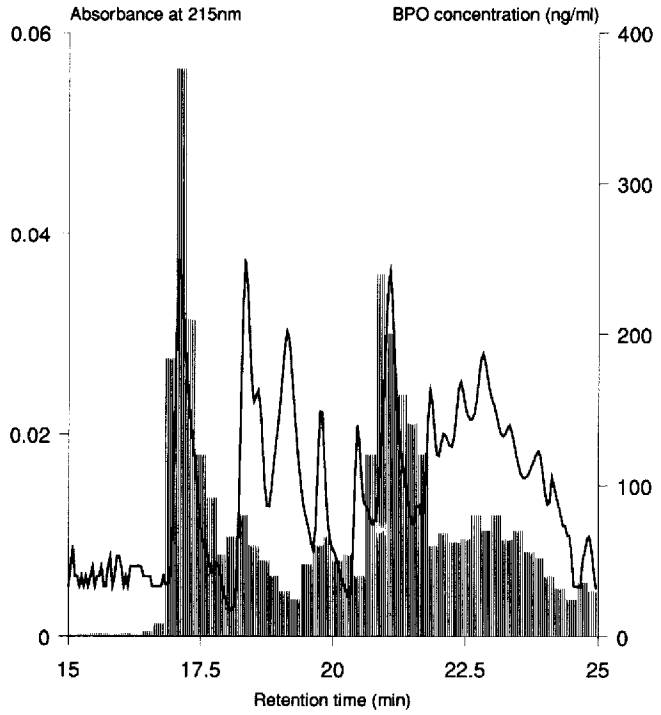

Fig. 2. RP-HPLC separation of the retained fraction on the immunoaffinity column. Columns and running conditions as in Fig. 1. Solid line, absorbance at 215 nm; hatched peaks, BPO concentration in each fraction.

peaks with retention times of 12.0 min (Pep 12) and 15.0 min (Pep 15). The sequence of Pep 21 corresponds to that of fragment 198–205.

Pep 12: $\underrightarrow{\text{Asp}_{187}}\text{–}\underrightarrow{\text{Glu}_{188}}\text{–}\underrightarrow{\text{Gly}_{189}}\text{–}\rightarrow\text{–}\underrightarrow{\text{Ala}_{191}}\text{–}\underrightarrow{\text{Ser}_{192}}\text{–}\underrightarrow{\text{Ser}_{193}}\text{–}\underrightarrow{\text{Ala}_{194}}\text{–}\underrightarrow{\text{Lys}_{195}}$

Pep 15: $\underrightarrow{\text{Ala}_{191}}\text{–}\underrightarrow{\text{Ser}_{192}}\text{–}\underrightarrow{\text{Ser}_{193}}\text{–}\underrightarrow{\text{Ala}_{194}}\text{–}\rightarrow\text{–}\underrightarrow{\text{Gln}_{196}}\text{–}\underrightarrow{\text{Arg}_{197}}$

Pep 21: $\underrightarrow{\text{Leu}_{198}}\text{–}\rightarrow\text{–}\underrightarrow{\text{CMCys}_{200}}\text{–}\underrightarrow{\text{Ala}_{201}}\text{–}\underrightarrow{\text{Ser}_{202}}\text{–}\underrightarrow{\text{Leu}_{203}}\text{–}\underrightarrow{\text{Gln}_{204}}\text{–}\underrightarrow{\text{Lys}_{205}}$

No amino acid could be detected in the fourth cycle of Pep 12 and in the fifth cycle of Pep 15 during the sequencing whereas BPO was found in these fractions. These data indicate that binding of BPO occurs at Lys 190 and Lys 195.

It should be emphasized that no tryptic cleavage occurs on a penicilloylated lysine and that lysine 190 and 195 are never found penicilloylated at the same time (or on the same peptide). On Pep 12 only Lys 190 is penicilloylated and not Lys 195, whereas when Lys 195 is penicilloylated (on Pep 15) Lys 190 is not penicilloylated. The peptide 187–197 where both lysine 190 and 195 could be penicilloylated is never observed.

In Pep 21 no amino acid is detected at the second cycle of the sequencing while BPO is found in the corresponding fraction, proving that the BPO binding site is located on lysine residue 199.

DISCUSSION

The *in situ* derivatization of the prepacked Ultraffinity-EP column is very easy and very fast. This column can also be used for the preparation of several milligrams of proteins in addition to the isolation of micro amounts of peptides. For the purification of antipenicilloyl antibodies, a large amount (30 mg) of HSA–BPO conjugate has been immobilized. This derivatized column permitted in each run (*e.g.*, 30 min), 6 mg of immunoglobulins to be purified from about 10 ml serum. The same type of column can also be derivatized with a much smaller amount of ligand. Only 13 mg of antipenicilloyl antibodies were used to prepare the immunoaffinity column.

In the preparation of the affinity column no chemical agent was used for blocking excess reactive groups on the Ultraffinity support, although this technique is classically used in low-pressure chromatography to prevent irreversible loss of sample materials on remaining active sites. We only verified that such a procedure was not necessary in the present instance as the BPO recovery was good and the non-specific binding of protein material on the column was small. In HPLC the elution time is much shorter than that in low-pressure chromatography, so that the contact between the sample material and the support might be too short to allow a non-specific reaction with the formation of a covalent bond. This would not be a difficulty when high specificity and affinity occur between the phase and the ligand such as in an antigen–antibody binding.

All the penicilloylated peptides appeared to bind to this column as no BPO was detected in the non-retained fraction. The recovery of these peptides was very good, as 95% of the amount of penicilloyl loaded were obtained in the fractions eluted from the reversed-phase column. Moreover, in order to establish the effect of the previous steps (*i.e.*, CNBr cleavage, subsequent trypsin treatment and heating) on the binding of BPO to protein fragments, total BPO was determined in each of these different steps in the overall procedure.

CNBr cleavage is known to occur specifically on methionine residues, which should not affect BPO bound to lysine residues. No data are available concerning the stability of the pseudo peptidic BPO–protein bond in formic acid, but under the acidic conditions in which sequencing is operated (TFA, HCl and heating) the bond appears to be stable and no BPO is lost. However, during the repeated steps of samples drying, evaporation of residual CNBr and formic acid and then redissolution of the dried hydrolysate, part of the protein material is lost. This loss (*ca.* 40%) is accompanied by the same loss of total BPO. The BPO recovery is therefore not complete during the CNBr cleavage but no particular peptidic fragment or binding site appears to be specifically involved.

Trypsin cleavage occurs on peptidic bonds involving a lysine or an arginine residue but not on pseudo peptidic bonds such as BPO–Lys. This was checked using a standard conjugate, *i.e.*, BPO–ε-aminocaproate. After trypsin hydrolysis, performed under the experimental conditions, more than 80% of the intact conjugate was recovered, which can be considered to be quantitative. Moreover, during the separation and identification of penicilloylated peptides, cleavage never occurred on a BPO–Lys. Trypsin cleaves neither the peptidic bond of penicilloylated lysine residue nor the pseudo peptidic BPO–lysine bond.

The chromatograms of the entire digest and of the peptides which were not

bound by the immunoaffinity column were nearly identical. This indicates that the non-specific binding of peptides was very low. However, the chromatogram of the retained fraction observed at a much greater sensitivity (*e.g.*, 0.06 a.u.f.s.) showed that some peptides which were bound to the immunoaffinity column did not contain a BPO group as detected by EIA. These peptides only represent a few percent (1–2%) of those injected. Janis and Regnier [7] also observed a non-specific binding of peptides on a covalently immobilized immunoaffinity column. This non-specific binding of peptides was probably more important than in our study, as they observed significant differences between the chromatogram of the non-retained peptides and the chromatogram of the entire tryptic digest. They indicated that treatment with Tween 20 of the immunoaffinity column decreased the non-specific binding of peptides but did not totally eliminate it. The non-specific binding of peptides is common in affinity chromatography and it is necessary to check the presence of hapten on peptides which bind to the IAC by a specific test applied subsequently.

However, the immunoaffinity step permits most of the non-penicilloylated peptides which represent about 98% of the peptides in the entire digest to be discarded. In a previous study, the isolation and separation of penicilloylated peptides from the tryptic digest of HSA fragment C needed three steps of HPLC purification. The first was a reversed-phase separation at pH 7.0. Each of the three penicilloylated fractions was then purified by ion-exchange chromatography and a third step of purification and desalting was done by reversed-phase chromatography using TFA–acetonitrile–isopropanol as the eluent. In each step all the eluted fractions must be collected and analysed for BPO. This means a total of seven chromatographic analyses and of about 700 samples to be analysed for BPO. Taking into account drying steps, dilution steps, etc., it took more than 6–7 days to obtain the purified penicilloylated peptides.

Moreover, it must be noted that very often the contamination of one peptide by another is only detected in the last step of the analysis during the identification. An entirely new procedure for the separation, detection and identification of each BPO peptide is then necessary, which means much more work. Using the dual column system, the same three penicilloylated peptides were obtained in a single step.

As the HSA molecule cannot be digested directly by trypsin because of its compact structure, we used several steps for its digestion. The CNBr cleavage permitted the molecule to be cut into three fragments and then to obtain tryptic digests containing a smaller number of peptides As the penicilloylated peptides represent only a few percent (1–2%) of the peptides, it is better to work on hydrolysates which are not too complex in order to decrease the proportion of non-specific contamination and the risk of eluting several peptides at an identical retention time.

The tryptic hydrolysates of the CNBr fragments A and B of HSA were analysed by this methodology [12] and three new binding sites for penicilloyl groups were unequivocally established: Lys 432, Lys 541 and Lys 545. It is worth noting that the same sites have been identified for the three *in vivo* and for the *in vitro* penicilloylated albumins, hence BPO-binding sites appear to be located only on lysine residues and only six different Lys residues out of a total of 59 can be penicilloylated.

As noted, all these potential binding sites cannot be penicilloylated at the same time (*e.g.*, on the same albumin molecule). This explains why six sites could have been detected while the measured average penicilloylation rate among the total population of penicilloylated albumin molecules was "only" two or three BPO groups per mole of HSA.

The data obtained give indications of the possible mechanism in penicillin allergy. A nucleophilic attack of the drug and then a covalent binding of BPO groups to proteins, *e.g.*, serum albumin, are necessary for the formation of the allergenic metabolite. However, this binding needs particular amino acid sequences and/or conformation of the protein molecule that are constant and somehow universal as they have also been described in several β-lactamases and protein-binding proteins [13].

It can be observed that four BPO–Lys out of six are located near serine from which they are separated by two amino acids. This position represents the configuration in which in an α-helix, Lys and Ser residues are the nearest. These results suggest a cooperation between these two residues in the mechanism of formation of the allergen metabolite of penicillin and therefore support the mechanism of penicilloylation proposed by Yamana *et al.* [14].

The BPO-binding sites are very few, located in small regions of the albumin molecule where they are concentrated, which certainly limits their bioavailability for specific antibodies. Some of them are not specific for penicillin: Lysine 199 is site of glycosylation, acetylation and aspirin binding.

All these considerations could suggest that under normal conditions the reacting molecules could rather be masked inside the protein carrier which would protect the organism against immunological disorders. Diseases and therapeutic, nutritional or environmental factors could unmask these determinants. Competition between ligands and modifications of the spatial structure of albumin can increase the number, the presentation and the chemical reactivity of reacting sites and thus permit an allergic process to be triggered.

CONCLUSION

The combination of affinity and RP-HPLC methods provides a rapid technique for the analysis of the binding sites of a hapten and more generally of any ligand covalently bound to a proteic carrier, and thus it provides a methodological approach for studying mechanisms of formation of allergenic determinants from drugs. The sensitivity of this association permits the analyses of very small amount of samples (*ca.* 100–200 μl of serum) which is especially useful for clinical studies in man.

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