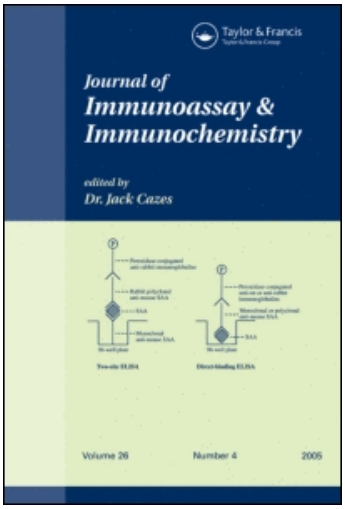

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

Enzyme Immunoassay of Benzyl Penicilloyl (BPO) Groups Using Acetylcholinesterase as Label. Application to the Study of the BPO-Binding Sites on Albumin

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To cite this Article Wal, Jean-Michel , Yvon, Mireille , Pradelles, Philippe and Grassi, Jacques(1991) 'Enzyme Immunoassay of Benzyl Penicilloyl (BPO) Groups Using Acetylcholinesterase as Label. Application to the Study of the BPO-Binding Sites on Albumin', Journal of Immunoassay and Immunochemistry, 12: 1, 47 — 64
To link to this Article: DOI: 10.1080/01971529108055056
URL: http://dx.doi.org/10.1080/01971529108055056

JOURNAL OF IMMUNOASSAY, 12(1), 47-64 (1991)

ENZYME IMMUNOASSAY OF BENZYL PENICILLOYL (BPO) GROUPS USING ACETYLCHOLINESTERASE AS LABEL. APPLICATION TO THE STUDY OF THE BPO-BINDING SITES ON ALBUMIN

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ABSTRACT

Benzyl penicilloyl groups (BPO) derive from penicillin G by cleavage of the β lactam ring ; they covalently bind to proteins to give conjugates which have lost all antibiotic properties but are considered as the major allergenic determinants in penicillin allergy. A solid-phase Enzyme Immuno Assay (EIA) of BPO groups in different biological fluids is described. It is a competitive immunoassay using acetylcholinesterase as label. In all biological fluids, very low non-specific binding values are observed. The sensitivity and the precision of the assay are good since ca. 0.5 ng/ml can be measured with a coefficient of variation less than 10 %. Cross reactions between BPO and penicillin or penicillin derivatives are nil or very low. This assay is more sensitive, much more rapid and easier to handle than the other methods available and is thus suitable for routine determinations. In association with reversed-phase high performance liquid chromatography this EIA has allowed an initial investigation of the location of BPO-binding sites on micro quantities of serum albumin (ca. 1 mg) from penicillin treated patients.

INTRODUCTION

Allergic accidents may occur in humans after penicillin therapy (1), or after consumption of food products, e.g. milk, from penicillin-treated animals (2). Numerous studies have emphasized the major role of benzyl penicilloyl (BPO)-protein conjugates as allergenic determinants. These conjugates derive from penicillin by cleavage of the β lactam ring and formation of a covalent bond between the carbonyl of BPO and ϵ amino groups of proteins (3-5). As these allergenic compounds have lost all antibacterial activity, they cannot be detected by conventional microbiological assay of penicillin. A radioimmunoassay (RIA) has been previously described for the specific detection of minute amounts of these BPO groups in biological fluids (6). It has been used to demonstrate that they are actually formed *in vivo* in humans (7) and in food-producing animals (8, 9) after penicillin therapy, and that they are present essentially as BPO-serum albumin conjugates.

The purpose of this work was to describe an alternative method that is more sensitive, rapid and easier to handle than the corresponding RIA for BPO groups using ^{125}I-labelled antigen. It should allow reliable routine determinations of trace amounts of BPO in biological fluids (e.g. serum, urine, milk). In addition, this would allow very sensitive detection of BPO binding to albumin molecules and determination of the binding site(s), using micro quantities of serum albumin from penicillin-treated patients.

Since the development of RIA other immunoassays have been proposed. Isetta et al. (10) developed an Enzyme Immuno Assay (EIA) where β-galactosidase-BPO conjugates were used as tracer. This assay was rather less sensitive than RIA (B/Bo 50% = 20 ng/ml vs 10 ng/ml). Moreover, the labelled conjugate appeared unstable, a progressive dissociation being observed as a function of time. A virus-enzyme immunoassay has been described by Mamas and Dray

(11), in which the tracer was a penicilloylated T_4 bacteriophage. Detection was sensitive but not suitable for routine assay. More recently, Lapresle and Lafaye (12) have proposed an Enzyme-Linked Immunosorbent Assay (ELISA) using alkaline phosphatase as label. The sensitivity of this assay was similar to that of RIA but the major characteristics (e.g. precision, specificity, reproducibility) were not characterized and the validity of the measurements performed was not demonstrated.

Here we describe a solid-phase EIA using acetylcholinesterase (AChE) as label. This enzyme has already been successfully used in EIA of several haptens and antigens (13-17). This is a conventional competitive immunoassay based on the use of an AChE-BPO conjugate and of a specific anti-BPO antiserum. The assay is performed in microtiter plates coated with a second antibody which ensures the separation process.

Associated with reversed-phase high performance liquid chromatography (HPLC) of a tryptic digest of penicilloylated human serum albumin (HSA), this EIA has allowed an initial investigation of the location of BPO-binding sites on the albumin molecule.

MATERIALS AND METHODS

Unless otherwise specified all chemicals were from Sigma.

Acetylcholinesterase (AChE : EC.3.1.1.7.) from Electrophorus electricus was prepared and purified as previously described (13).

AChE activity was measured by the colorimetric method of Ellman (18) adapted as previously described (13). One Ellman unit is defined as the amount of enzyme inducing an increase of 1 absorbance unit during 1 min., in 1 ml of Ellman's medium, for a 1 cm pathlength. It corresponds to about 8 ng of enzyme.

The BPO-AChE conjugate was prepared using an enzyme preparation essentially comprising the G4 form of AChE (for a

review of AChE molecular forms see 19), as described for other assays (13-17).

Specific anti-BPO rabbit antiserum was prepared as described by Wal et al. (6).

Solid-phase EIA was performed by using specialized Titertek microtitration equipment including an automatic plate washer (Microplate washer 120), an automatic dispenser (Autodrop) and a spectrophotometer (Multiskan MCC) set at 414 nm, all from Flow Laboratories. Microtiterplates were 96 F immunoplates I with certificate from Nunc.

HPLC was performed using a Waters Ass. chromatograph equipped with two M510 pumps, a WISP 712 automatic injector, and an M841 double beam UV detector set at 215 nm. Fractions were collected with a TDC 80 Microcol (Gilson).

Preparation of BPO-G4 AChE Conjugate

The BPO-G4 AChE conjugate was obtained by directly coupling the BPO groups to the enzyme.

0.4 nmol. of G4 AChE was added to 0.1 µmol. of penicillin G (crystallized sodium benzyl penicillinate, Specia) in 0.2 ml of 0.1M borate buffer pH 9.2. The reaction was allowed to proceed overnight at 22°C. The reaction mixture was then diluted two fold in the following buffer (EIA buffer) : 0.1M phosphate buffer pH 7.4, 0.4M NaCl, 10^{-3}M EDTA containing 0.1 % bovine serum albumin and 0.01 % sodium azide. The enzymatic activity was determined and the purification was performed by HPLC on a Superose 6™ column (Pharmacia) with EIA buffer as eluent. Fractions (0.4 ml) were collected in 1 ml of EIA buffer. The enzyme activity in each fraction was measured for a 5 µl aliquot in a microtiterplate well with 0.2 ml of Ellman's reagent. Fractions containing AChE activity corresponding to the G4 form of the enzyme were pooled. The immunoreactivity was checked in the presence of different dilutions of anti-BPO antiserum and the pool (e.g. BPO-G4 AChE)

was stored at -80°C. No significant loss in enzyme activity was observed during the overall procedure.

EIA Procedure

96-well microtiterplates were coated with swine anti-rabbit (SaR) IgGs as previously described (13), and stored at +4°C in presence of EIA buffer.

Just before use, the plates were washed (washing buffer : 0.01M phosphate buffer pH 7.4 containing 0.05 % Tween 20) and EIA was performed as summarized in Fig. 1. 50 µl of each reagent (e.g. tracer, antiserum and standard or sample) at appropriate dilutions were added. Enzymatic tracer was habitually used at a concentration of 1 Ellman unit/ml. BPO ϵ amino caproate (Sigma) was used as standard. Non-specific binding was determined in separate wells containing 100 µl of buffer, serum, urine or milk and 50 µl of BPO-AChE tracer during immunoreaction.

The results were expressed in terms of B/Bo x 100 where B and Bo represent the absorbance values measured on the bound fractions in the presence (B) or absence (Bo) of BPO competitors (e.g. BPO-ϵ amino caproate for the standard dose-response curves, BPO-HSA or BPO-peptides for determinations in unknown samples). Standard curves and calculations of sample concentrations were performed using a linear log-logit transformation.

All concentrations mentioned in this paper are initial values and refer to the concentration of the reagent in the 50 µl volume, before mixing with other reagents.

The sensitivity of EIA was characterized either by the amount of standard BPO inducing 50 % inhibition of Bo (B/Bo = 50 %), or by the "minimum detectable concentration", defined as the concentration of standard inducing a significant lowering of Bo (i.e. Bo-3 standard deviations).

Cross-reactions (CR) between BPO and penicillin derivatives were determined by comparing standard curves obtained with the

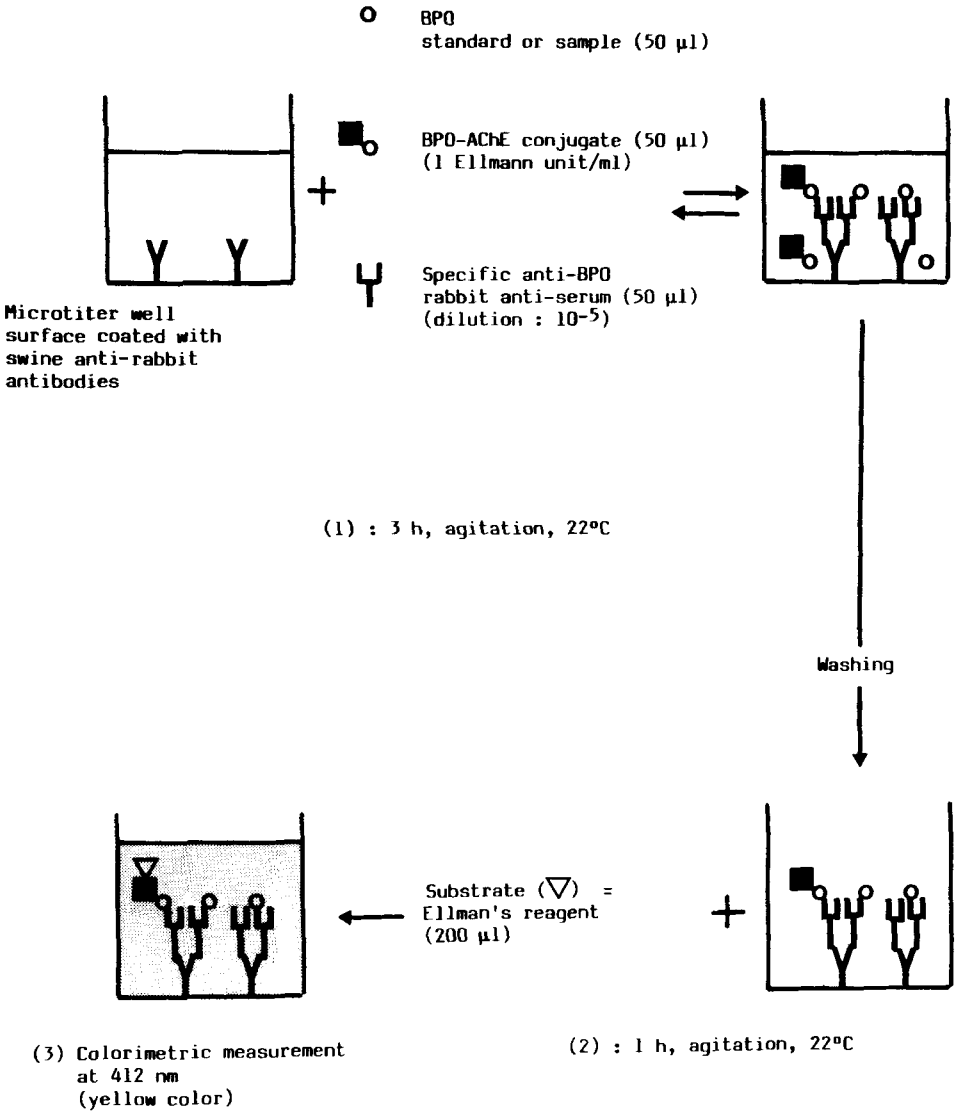


FIGURE 1. EIA procedure

different haptens. Cross-reactivity coefficients were calculated using the Abraham criterion (20)

$$CR\ \% = \frac{(B/BO = 50\ \%)\ \text{observed with BPO } \epsilon\text{-amino caproate}}{(B/Bo = 50\ \%)\ \text{observed with analog}} \times 100$$

Obtention of Penicilloylated Human Serum Albumin

Control human serum albumin (HSA) was obtained from Centre National de Transfusion Sanguine. Free fatty acids were removed according to Chen (21). This sample was treated and analyzed under the same conditions as described below.

Sera were obtained from three patients in the Institut Pasteur Hospital, who received intravenously 50 x 10^6 IU Penicillin G per day during 3-4 weeks. The sera were collected within the first week after cessation of treatment and the penicilloylated albumin (HSA-BPO) which presents a faster electrophoretic mobility was separated by ion exchange chromatography on DEAE Sephadex (7). For each serum c.a. 1 mg of HSA-BPO was thus preparated . 1 % aliquot (c.a. 150 pmol HSA-BPO) was kept for BPO determinations.

CNBr cleavage of penicilloylated-albumins, separation of fragments and determinations of penicilloylated peptides

Control HSA and penicilloylated serum albumins obtained *in vivo* were hydrolysed by using cyanogen bromide (CNBr) in a 70 % formic acid solution according to (22). After 24 hours incubation, the reagents were removed by freeze drying.

Separation of fragments was performed by reversed-phase high performance liquid chromatography (HPLC) using a Waters Ass. Chromatographic System. The CNBr digests were dissolved in 1 ml solvent A (0.05 % aqueous solution of trifluoroacetic acid (TFA)). 50 µl were injected onto an Aquapore RP-300 column (7 µm, 30 x 4.6 mm I.D. ; Brownlee), equilibrated in a mixture of 50 % solvent A and 50 % solvent B (acetonitrile/2-propanol/0.05 % TFA in water v/v/v).

Elution of peptides was achieved with a 30 min linear gradient from 50 to 100 % solvent B at a flow-rate of 1 ml/min. All runs were performed at room temperature and peptides were detected at 215 nm. Fractions were collected every 30 sec using a TDC 80 microcol (Gilson), and EIA determinations of BPO were performed on each of these fractions.

The normal albumin digest chromatogram shows 3 fragments which were identified to the classical CNBr fragments $C_{124-298}$, $A_{299-585}$ and B_{1-123} by their amino acid analyses and by the determination of the C-terminal and N-terminal amino-acids. Their retention time (RT) were 7.5, 9.4 and 11.8 min. respectively. Fragment C is constituted by a single peptidic chain, while fragments B and A are respectively composed of two and four chains held together by S-S bonds.

The 4 peptidic chains of fragments A were separated as follows. After an overnight reduction in urea and a 10 fold molar excess of dithiothreitol, an alkylation of the SH-groups of the fragments A was performed using iodoacetic acid according to Swenson (23), the mixture was then analysed by RP-HPLC using the same column as described. The elution was achieved by a 30 min linear gradient from 0 to 100 % of TFA in the acetonitrile-propanol-water solvent. 0.05 ml fractions were collected for the EIA-determinations of BPO groups.

The 4 peaks (RT 17.7, 18.9, 20.1 and 22.7 min.) observed with the normal serum albumin correspond to sequences 299-329, 549-585, 447-548 and 330-446 respectively, according to their amino acid compositions, and to the determinations of C-terminal and N-terminal amino-acids.

RESULTS AND DISCUSSION

EIA Characteristics

The standard curves obtained in EIA buffer and in different biological fluids (e.g. serum, urine and milk) are presented in

Fig. 2. Determinations were performed under the conditions described in Fig. 1 using different concentrations of BPO ϵ aminocaproate as standard. Anti-serum was used at a 10^{-5} dilution.

Even if differences in binding were observed in the different media (variations of Bo), the corresponding standard curves (expressed in term of B/Bo %) were very similar. The sensitivity of the assay was very good since B/Bo ≈ 50 % values between 1.3 and 2 ng/ml were observed in the different media. Accordingly, minimum detectable concentrations as low as 0.3 ng/ml could be calculated. This sensitivity is greatly superior to that observed with previously published RIA or EIA (10-20 ng/ml) (6, 10, 12). It is worth noting that very low non-specific binding values were observed (0.02 to 0.04 % of the total enzymatic activity introduced in the assay). The precision of the assay was also satisfactory as illustrated in Fig. 3 which indicates the imprecision profiles corresponding to the curves in Fig. 2. These profiles show that coefficients of variation close to or less than 10 % were observed from 0.3 to 10 ng/ml whatever the medium.

Cross-reaction between BPO and penicillin derivatives was nil or very low since cross-reactivity coefficients lower than 0.15 % and 0.6 % were determined with penicillin G and penicillenic acid respectively while no cross-reactivity was detected with D or DL benzyl penicillamine.

When conjugated to a protein, BPO groups seem to be recognized in the same way as standard BPO ϵ aminocaproate. This is illustrated in Fig. 4 where a dilution curve obtained with BPO-HSA conjugate and a standard curve are essentially parallel. This confirms the validity of EIA measurements on BPO-protein or BPO-peptide conjugates.

Study of the penicilloylated serum albumin fragments from penicillin-treated patients

Total BPO groups determined in each fraction of isolated and purified fast (e.g. penicilloylated) albumin from the different

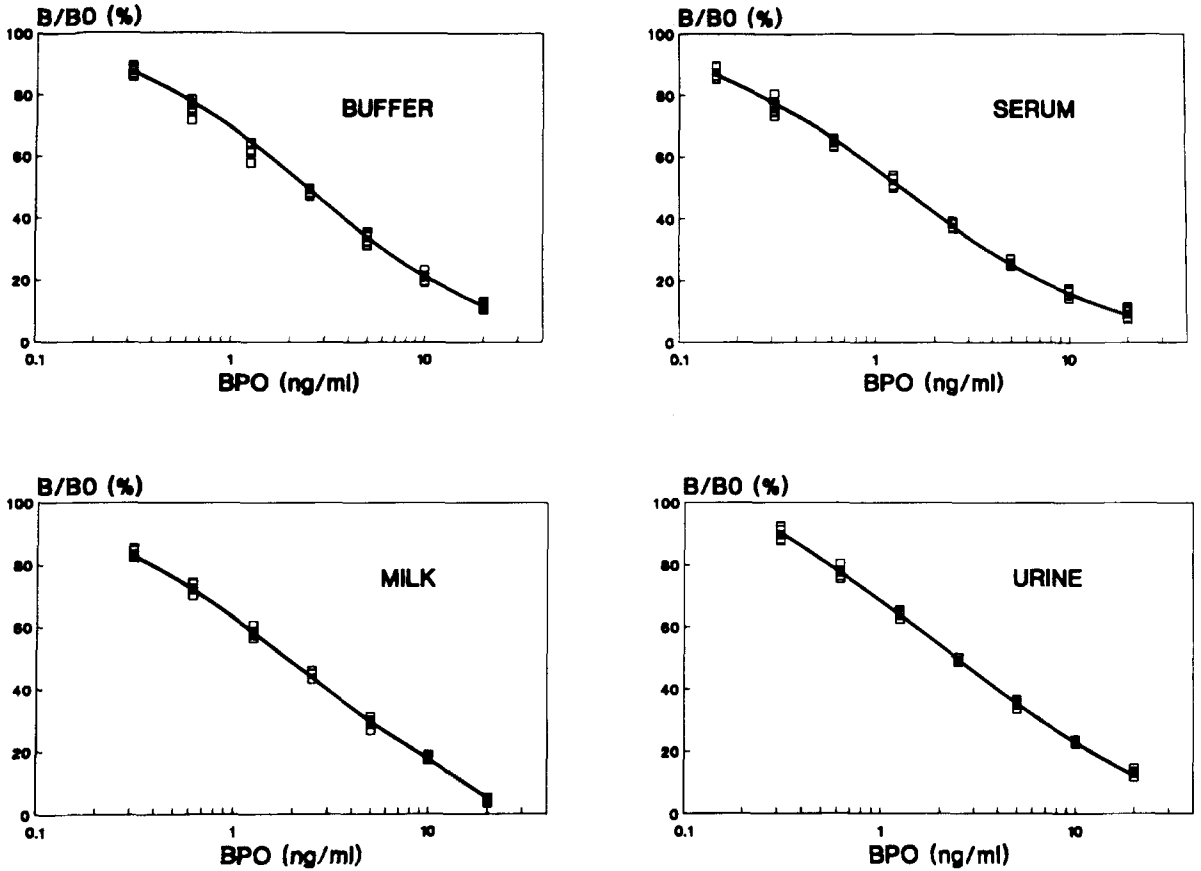


FIGURE 2. EIA standard curves obtained in different biological fluids. For each dilution of the standard antigen (BPO ϵ amino caproate) in buffer, serum, urine or milk, 6 measurements were made.

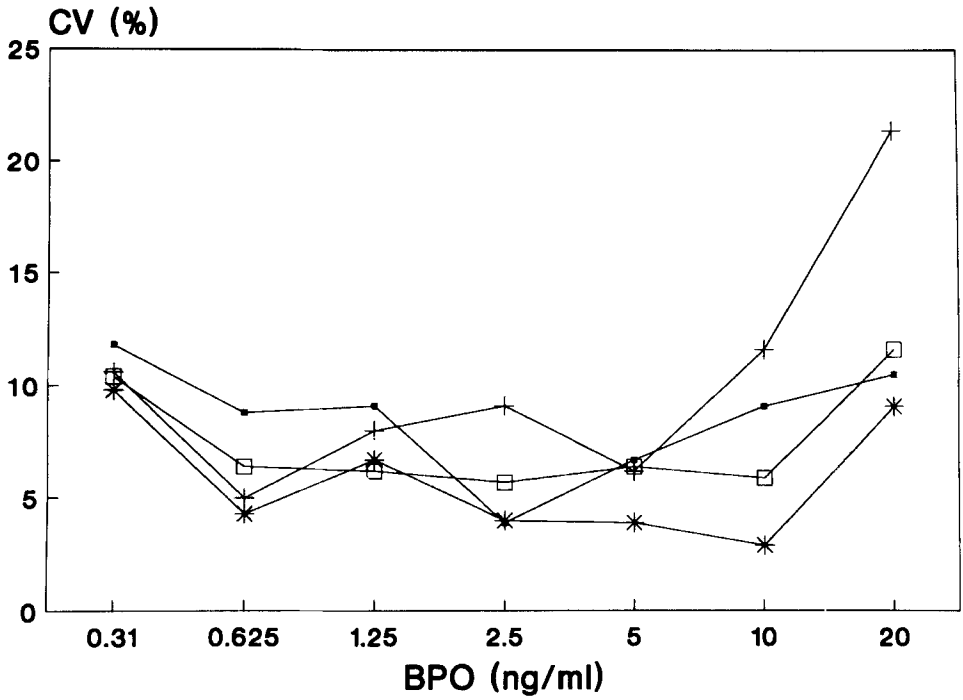


FIGURE 3. Imprecision profiles observed for EIA in different biological fluids. Buffer, ■ ; serum, + ; urine, ✳ ; milk, □ . The coefficients of variation (C.V. %) were calculated from 8 determinations of each dilution.

sera were 2.8-3.2 BPO per mol HSA as measured by EIA and confirmed by RIA (6,7) and by the classical penamaldate method (24).

A mean of 270 pmol BPO is recovered in the whole CNBr digests. This c.a. 40 % loss of BPO present in the conjugates is accompanied by the same loss of protein material due to the different steps of samples drying, evaporation of residual CNBr and formic acid, and then redissolving the dried hydrolysates.

The BPO recovery is therefore not complete during the CNBr cleavage but no particular peptidic fragment or binding site appears to be specifically involved.

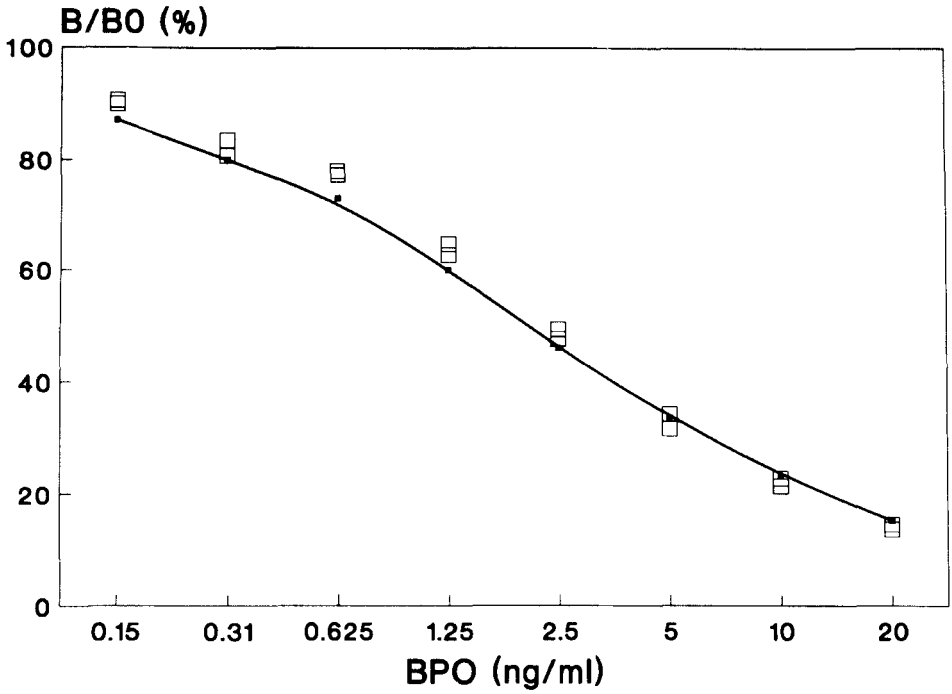


FIGURE 4. Dose-response curve established with two-fold successive dilutions of BPO-HSA conjugate. Duplicate determinations of BPO were performed at different dilutions of BPO-HSA conjugate (□). Each dilution was calculated such that the amount of BPO (determined by RIA and by the penamaldate method) was the same as for the corresponding point of the standard curve established with BPO ϵ amino caproate (■).

The chromatographic pattern of CNBr fragments separation shown in Fig. 5 was similar for all the analyzed sera.

No BPO was found in the fragment B of any of the 3 sera.

In the three penicilloylated albumins, the peaks corresponding to the fragment C decrease while new peaks (RT 8.5 min) appear. Using amino acid analysis and N- and C- terminal amino acids determination, these peaks were identified as the fragment C which possess BPO groups.

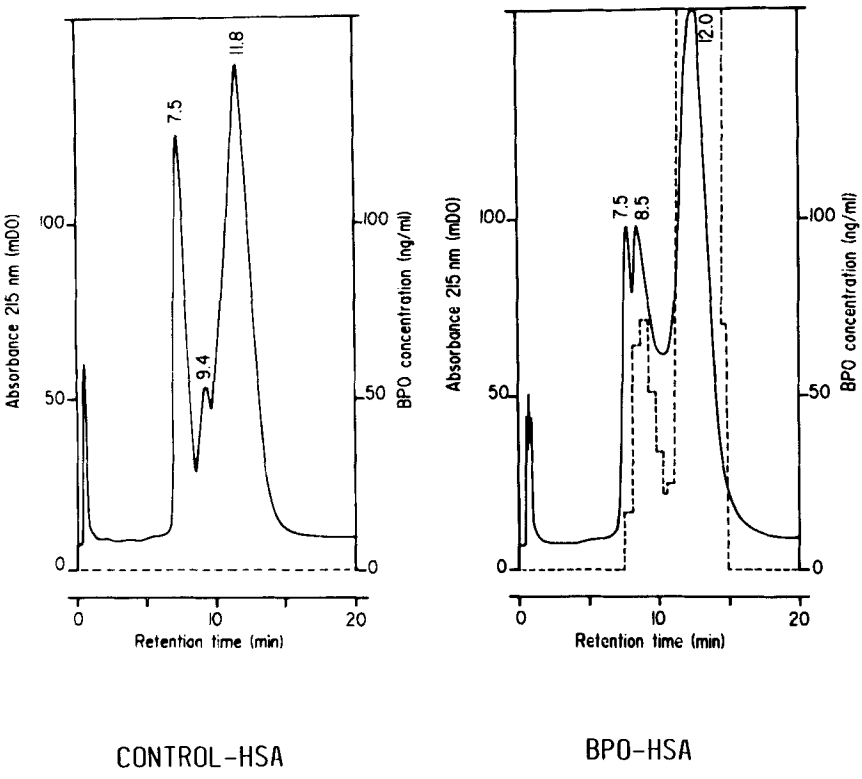
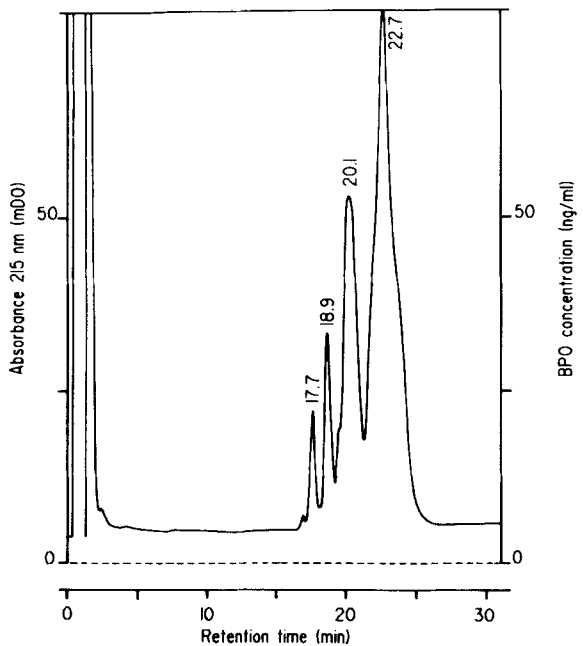


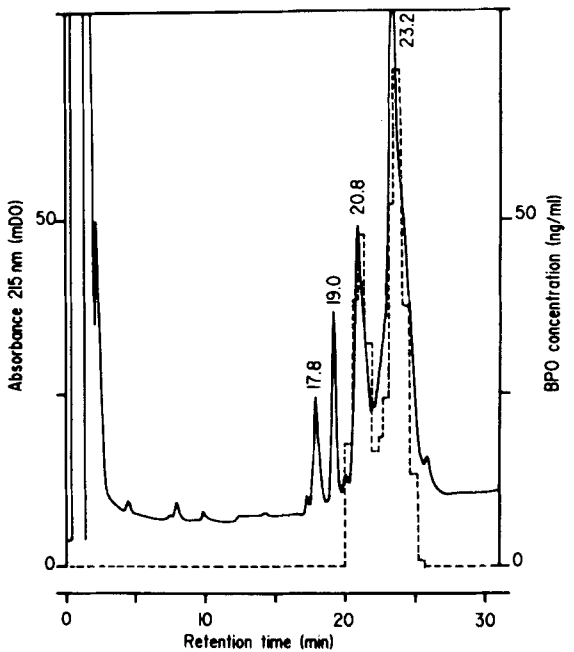
FIGURE 5. HPLC fractionation of the CNBr fragments from normal serum albumin and from *in vivo* penicilloylated albumin isolated from penicillin-treated patients serum. Absorbance (215 nm) due to peptides, ——— ; EIA determinations of BPO in the corresponding fractions, - - - .

The peaks corresponding to the fragment A (RT 12.0 min) are slightly shifted towards higher RT and were actually confirmed to be the fragment A which contains also BPO.

59 and 130 pmol were determined in the pooled fraction corresponding to fragment C and fragment A peaks respectively. The recovery of the HPLC separation is c.a. 67 %. 1/3 of the BPO is located on fragment C while 2/3 are located on fragment A.



CONTROL-HSA FRAGMENT A



BPO-HSA FRAGMENT A

FIGURE 6. HPLC-separation of the 4 peptidic chains from the CNBr fragments $A_{299-585}$ prepared from normal albumin and from penicilloylated albumin isolated from patients serum. Absorbance (215 nm) due to peptides, ———; EIA determinations of BPO in the corresponding fractions, - - - - .

HPLC analyses of reduced and S-carboxymethylated fragments A are shown in Fig. 6. The same pattern is observed with the samples prepared from the three different sera.

For each fragment A, two penicilloylated containing peaks were obtained which were slightly shifted toward higher RT : 20.8 and 23.2 min. The peptides eluted in these fractions were identified with sequence 447-547 and 330-446 respectively by their amino acid analyses and by the determination of the C- and N-terminal amino acids.

In the three fragments A obtained from the different penicilloylated serum albumins, the same sequences were found.

90 % recovery is observed during this separation 26 % of the BPO is located on peptide 447-547 and 74 % on peptide 330-446.

The whole procedure was performed on a control HSA sample ; no false positive result occured at any step of the analyses.

Binding sites of the allergenic determinant of Penicillin (BPO) on to albumin molecules thus appear to be multiple and the same location is found whatever the penicilloylated serum albumin comes from. This evidence of multiple binding sites confirms our previous observation on the electrophoretic heterogenicity of penicilloylated albumin (7) and the results from Lafaye and Lapresle (25) who demonstrated two major BPO-binding sites on fragments $C_{124-298}$ and $A_{299-585}$ of albumin from penicillin-treated patient.

CONCLUSIONS

The EIA for BPO described in this paper appears both precise and sensitive. Good sensitivity is essentially due to the advantageous properties of AChE, the very high turnover of which allows the preparation of conjugates with high specific activity, detectable in the attomole range (26). This has already been shown for other haptens or antigens (13-17). The good precision is linked to low non specific binding and to the use of solid-phase separation allowing virtually complete automation of the assay.

In association with HPLC, the EIA of BPO described here thus appears to be an improved immuno-analytical tool for studying the location of BPO-binding sites on albumin, and particularly on serum albumin from penicillin-treated patients and thus the mechanism of formation of the allergenic metabolite of Penicillin.

The sensitivity of the assay allows the use of micro quantities of experimental material (c.a. 1 mg penicilloylated protein). Most of the sample remains available for various multiple cleavages (e.g. using cyanogen bromide, trypsin, etc...), chromatographic fractionation and purification of the fragments, thus allowing identification of the peptide-bearing BPO group, since only 1 % of each fraction is necessary for PBO determinations at each step of this procedure.

Moreover, this EIA for BPO groups is better adapted than RIA for control routine determinations of penicilloylated residues in biological fluids from food-producing animals that might have been treated with penicillin. It is more sensitive and, in particular, much more rapid and easier to handle than ^{125}I-labelled tracer. The AChE-BPO tracer can be prepared in one large batch and stored at -80°C, since no loss of immunoreactivity or enzymatic activity has been observed during storage for several months.

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