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Development and validation of an indirect enzyme-linked immunosorbent assay (ELISA) for the nonsteroidal anti-inflammatory drug *S*-ibuprofen

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An indirect enzyme-linked immunosorbent assay (ELISA) was developed for the nonsteroidal anti-inflammatory drug (NSAID) *S*-ibuprofen. Conjugates for immunization were prepared by linking *S*-ibuprofen via the spacer 4-aminobutyric acid to bovine serum albumin as well as to a novel synthetic lipopeptide using the *N*-hydroxysuccinimide/dicyclohexylcarbodiimide method. Immunization with these immunogens was carried out in New Zealand rabbits. A poly-L-lysine-*S*-ibuprofen conjugate was used as a hapten-carrier for coating the surface of the microtiter plates with the hapten. Horseradish peroxidase labeled anti-rabbit IgG served as secondary antibody using hydrogen peroxide and ABTS as substrates. The characterization of the polyclonal antiserum with compounds of analogous structure demonstrated that the antiserum possesses a very high specificity for *S*-ibuprofen (cross-reactivity <0.14–1.4%). Additional cross-reactivity experiments using *R*-ibuprofen (cross-reactivity 50.5%), ibufenac (58%) and isopropylphenylacetic acid (6.4%) were carried out to obtain more detailed information about the antigenic recognition concerning the chiral center. The results indicated that the polyclonal antiserum possesses an additional antibody population, whose antigenic recognition did not contain the chiral center. The upper and lower limits of quantification of the developed ELISA were defined as 362 and 3.62 ng *S*-ibuprofen/ml, respectively, based on a 90% confidence interval.

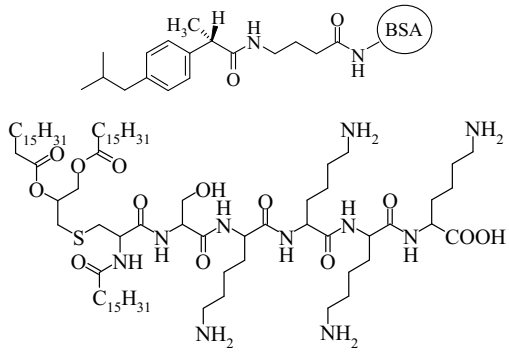
1. Introduction

The "profens" are an important group of NSAIDs. Over the past three decades, a wide range of profens have been used as analgesic and anti-inflammatory agents [1]. The realization that the pharmacological activity of many chiral NSAIDs resides in one or the other enantiomer led to the recent interest in assessing the therapeutic value of such enantiomers [2]. This concept is especially important with ibuprofen, which undergoes chiral inversion in humans via coenzyme A thioesters [3]. A quantitative determination of the enantiomers would allow a closer definition of the dose of active drug and therefore can be useful to avoid potential side effects [4].

A great variety of analytical methods exists for the quantitative determination of the ibuprofen enantiomers, e.g., HPLC- [5–8], GLC- [9], GC-MS- [10, 11] and NMR [12] methods. These methods all have in common that they require large-scale sample preparation. ELISA is the method of choice, since it combines high analytical sensitivity with simple sample preparation and a high sample throughput. The present ELISA was developed as an indirect, heterogeneous competitive assay, because this method possesses important advantages over the direct test, such as the high flexibility of the detection system due to the large number of commercially available secondary enzyme-labeled antibodies, and the possible detection of various antigens with a single enzyme-labeled anti species antibody [13–15].

2. Investigations, results and discussion

The aim of the present study was the development and validation of an indirect ELISA for the NSAID *S*-ibuprofen. To produce the specific *S*-ibuprofen antibodies required for the development of the ELISA, *S*-ibuprofen was linked to macromolecular carriers, since *S*-ibuprofen as a low molecular weight hapten is not able to induce an immune response. Two different carriers were used to prepare the immunization conjugates: BSA versus a novel synthetic lipopeptide Pam_3Cys-Ser-$(Lys)_4$-OH.



2.1. BSA-S-ibuprofen conjugates

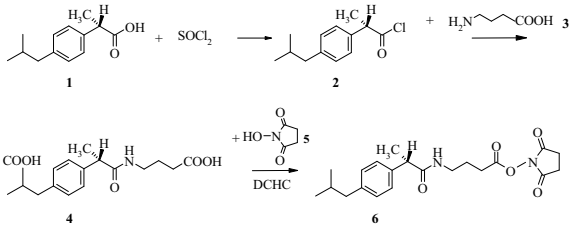
2.1.1. Introduction of a spacer to the hapten

The commonly used C4-spacer 4-aminobutyric acid was added to the hapten molecule in order to obtain a sufficient distance between the chiral center of the hapten and the carrier protein. The carboxyl groups of *S*-ibuprofen and *S*-ibuprofen-4-butyric acid were activated by means of the *N*-hydroxysuccinimide (NHS)/dicyclohexylcarbodiimide (DCHC) method (Scheme). To assure the purity and the preserved *S*-conformation of the obtained immunogens for the immunization, the resulting activated haptens were analyzed by HPLC. The purity of the obtained *S*-ibuprofen-4-aminobutyric acid-*N*-hydroxysuccinimide was higher than 99.5%. Using a chiral AGP column it was demonstrated that both *S*-ibuprofen-4-aminobutyric acid and its activated NHS-ester preserved their *S*-conformation.

2.1.2. Synthesis and characterization of BSA-hapten conjugates

Activated *S*-ibuprofen-4-aminobutyric acid and BSA were dissolved in dioxane/water (1 : 1), 1 M NaOH solution was added until the apparent dullness disappeared. The mixture was stirred for 24 h at room temperature and was then dialyzed against water to remove unbound *S*-ibuprofen and lyophilized. The assay was carried out at different molar hapten/BSA ratios to achieve the maximum labeling degree (Table).

Scheme



Synthesis of *S*-ibuprofen-4-aminobutyric acid-*N*-hydroxysuccinimide (**6**) using the *N*-hydroxysuccinimide (NHS)/dicyclohexylcarbodiimide (DCHC) method: First, the carboxyl group of S-ibuprofen (**1**) was activated using thionyl chloride. The resulting *S*-ibuprofen chloride (**2**) reacted with the spacer molecule 4-aminobutyric acid (**3**) to *S*-ibuprofen-4-aminobutyric acid (**4**), whose carboxyl group was activated using *N*-hydroxysuccinimide (**5**) to form *S*-ibuprofen-4-aminobutyric acid *N*-hydroxysuccinimide (**6**).

The resulting hapten-BSA conjugates of different molar hapten/BSA ratios were characterized with respect to the labeling degree of *S*-ibuprofen. In the case of the hapten-BSA conjugates, an HPLC method was used after performing total conjugate hydrolysis. The conjugates were incubated with 6 M HCl and for 24 h at 120 °C. *S*-ibuprofen was isolated by extraction with *n*-hexane/diethylether (8 : 2). Flurbiprofen was used as an internal standard, calibration curves were prepared in the range of 0.1 – 1 mg/ml. Due to the fact that ibuprofen is extensively bound to plasma proteins ($\geq$99%), an apparently high labeling degree was found, which consisted of non-covalently and covalently bound ibuprofen (Table).

Consequently, the hapten-BSA conjugates had to be treated with a chaotropic substance prior to the determination of covalently bound ibuprofen. Chaotropic ions are used for reversible controlled perturbation of the native structure of proteins [16], in the present study the conjugates were treated with a 3 M KSCN solution. Then the conjugates were dialyzed to remove reversibly bound ibuprofen and the chaotropic ions, the completeness of the dialysis was indicated using $FeCl_3$. The characterization of covalently bound *S*-ibuprofen was performed as described above. Up to a molar ratio of 60 : 1 hapten/BSA, an increase of labeling degree was noticed, while the molar ratios 120 : 1 and 240 : 1 already indicated a saturation effect (Table).

Table: Synthesis of immunization conjugates of different molar hapten (*S*-ibuprofen-4-aminobutyric acid-*N*-hydroxysuccinimide)/BSA ratios

Molar hapten/BSA ratio	Apparent labeling degree	True labeling degree
15 : 1	16.8	1.8
30 : 1	28.5	6.3
60 : 1	51.9	14.4
120 : 1	65.1	19.8
240 : 1	82.9	22.8

Labeling degrees were determined by HPLC, the true labeling degree was obtained after treatment with chaotropic ions

2.2. Lipopeptide-S-ibuprofen conjugate

2.2.1. Synthesis and characterization of the lipopeptide-S-ibuprofen conjugate

Analogues of the N-terminus of bacterial lipopeptides, consisting of Pam_3Cys (*N*-palmitoyl-*S*-[2,3-bis-(palmitoyloxy-)-(2-*R*,*S*)-propyl]-(*R*)-cysteine) attached to one to five further amino acids, represent potent novel immunoadjuvants in mice, rabbits, and other species. When used together with antigens, Pam_3Cys-peptides markedly enhance the antigen-specific humoral or cellular immune response. They are non-toxic, non-pyrogenic, and do not induce tissue damage when injected [17]. Lipopeptides, covalently coupled to low molecular weight haptens, are able to elicit high antigen-specific antibody titers in mice and rabbits.

In the present study, *S*-ibuprofen succinimide as a low molecular weight hapten was linked to (*S*-[2,3-bis-(palmitoyloxy-)-(2-*R*,*S*)-propyl]-*N*-palmitoyl-(*R*)-Cys-(*S*)-Ser-(*S*)-Lys_4-OH [(Pam_3Cys-Ser-$(Lys)_4$]). The synthesis of *S*-ibuprofen succinimide, the examination of its purity and of the preservation of the *S*-conformation were carried out as described above (2.1.2.). Pam_3Cys-Ser-$(Lys)_4$ was dissolved in water, *N*-ethylmorpholine and *S*-ibuprofen succinimide (dissolved in 1,4-dioxane) were added, and

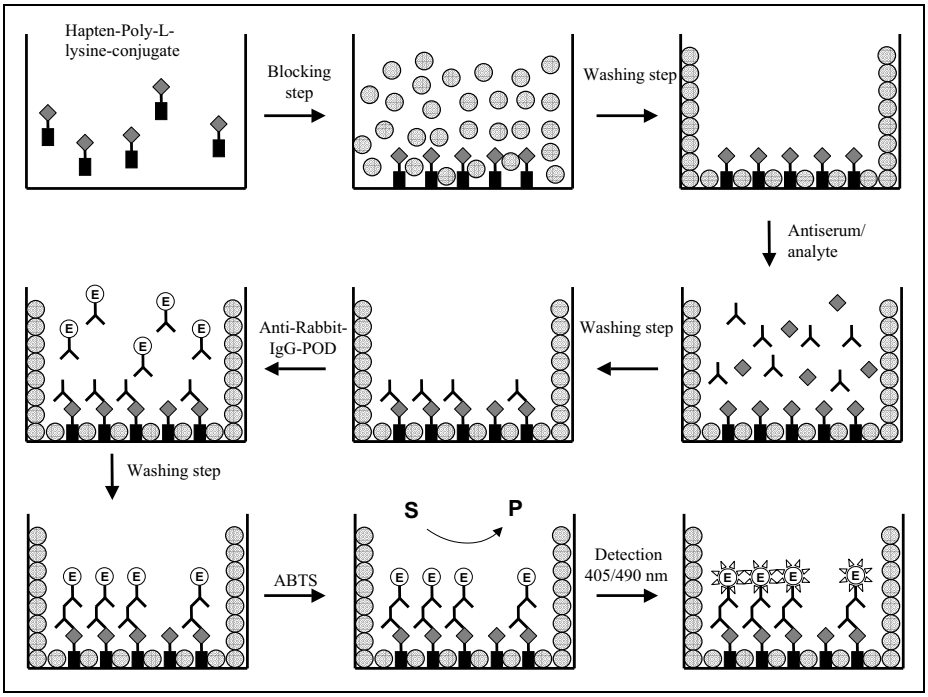


Fig. 1:
Principle of the used indirect heterogeneous competitive ELISA: The first step consists of coating the hapten *S*-ibuprofen with the help of the carrier protein poly-L-lysine to the surface of microplate wells. Due to the fact of remaining free binding sites, an additional blocking step is required and followed by the incubation step with the sample (antiserum). If the sample also contains antigen a competition between solid phase bound antigen and antigen in the sample will decrease the antibody concentration which in turn is detected by a horseradish peroxidase-labeled anti-rabbit IgG as a secondary antibody using hydrogen peroxide and ABTS as a substrate

the mixture was stirred for 144 h at room temperature. The resulting precipitate was washed 3 times with cold 1,4-dioxane/H_2O (1 : 1), reconstituted in *tert*.-butanol, and lyophilized. The characterization of the lipopeptide-hapten conjugate was performed by MALDI-MS and ^1H NMR. The resulting labeling degree was found to be 2–4 mol hapten/mol lipopeptide.

2.3. Immunization and isolation of the γ-globulin fractions

New Zealand rabbits were treated with a mixture of the synthesized protein-hapten conjugates and *Freund's* adjuvant. The immunization was carried out with BSA-hapten-conjugates of different molar BSA/hapten ratios as well as one lipopeptide-hapten conjugate. The γ-globulin fractions were isolated by ion-exchange chromatography on DEAE-cellulose. The purity of the resulting γ-globulin fractions were examined by immuno electrophoresis using the method of Grabbar and Williams [18].

2.4. ELISA

In this study an indirect heterogeneous competitive enzyme immunoassay was developed. In heterogeneous assays, the enzyme activity is unaltered by antigen-antibody reaction, thus, reacted antibody or antigen must be separated from the unreacted component. Solid phases, to which one of the immuno reactants is attached, enable a fast and complete separation from those in the supernatant. Figure 1 shows every single step of the developed ELISA, each step is followed by a washing step to separate unbound from bound components. First of all, *S*-ibuprofen is linked with the help of the carrier protein poly-L-lysine to the surface of microplate wells. Because of the fact that the coating of the surface is not complete, free binding sites remain and an additional blocking step is necessary. The following step includes the incubation with the antiserum. If, however, the added sample also contains antigen, a competition between the solid phase bound antigen and the antigen in the sample will decrease the antibody concentration, which finally leads to a decrease of the signal. The degree of inhibition of the detector reaction is inversely proportional to the amount of antigen in the test sample. The detector reaction was performed by a horseradish peroxidase-labeled anti-rabbit IgG as a secondary antibody using hydrogen peroxide and ABTS (2,2′-azino-di-[3-ethylbenzthiazoline sulfonate]) as a substrate.

During the development of this hapten-specific ELISA, each step was optimized to eliminate unspecific side-reactions.

2.4.1. Coating of the surface of the microtiter plates with hapten

The fixation of the used hapten to the surface of microplate wells was carried out with different carrier proteins. Due to the fact that polyclonal antisera contain hapten-specific antibodies as well as unspecific antibodies (e.g., antibodies against the carrier protein used for immunization), potential cross-reactivity of the antisera with the carrier proteins was analyzed (Fig. 2). Commonly used carrier proteins for linkage to solid phases are BSA and poly-L-lysine. The antisera derived from the BSA- and lipopeptide-hapten conjugates were named *AS BSAγ* and *AS lipoγ*, respectively. As expected, *AS BSAγ* showed cross-

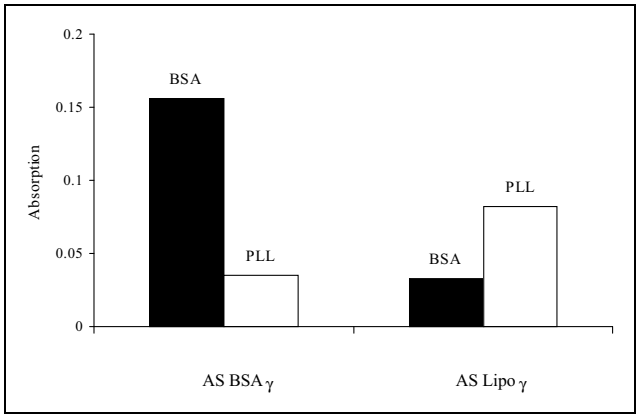


Fig. 2: Cross-reactivity of *AS BSAγ* and *AS lipoγ* with different carrier proteins

reactivity with BSA, since BSA served as a carrier protein in the immunization conjugate. *AS lipoγ* showed minor cross-reactivity with poly-L-lysine, which may be the result of a reaction with antibodies directed against the lysine residues of the Pam_3Cys-Ser$(Lys)_4$-peptide. Therefore, we used poly-L-lysine to link the hapten to the solid phase for the establishment of the ELISA with *AS BSAγ* and BSA for the testing of *AS lipoγ*.

2.4.2. Blocking of free remaining sites

Nonspecific binding to the solid phase limits sensitivity and should be as low as possible. Thus, an additional

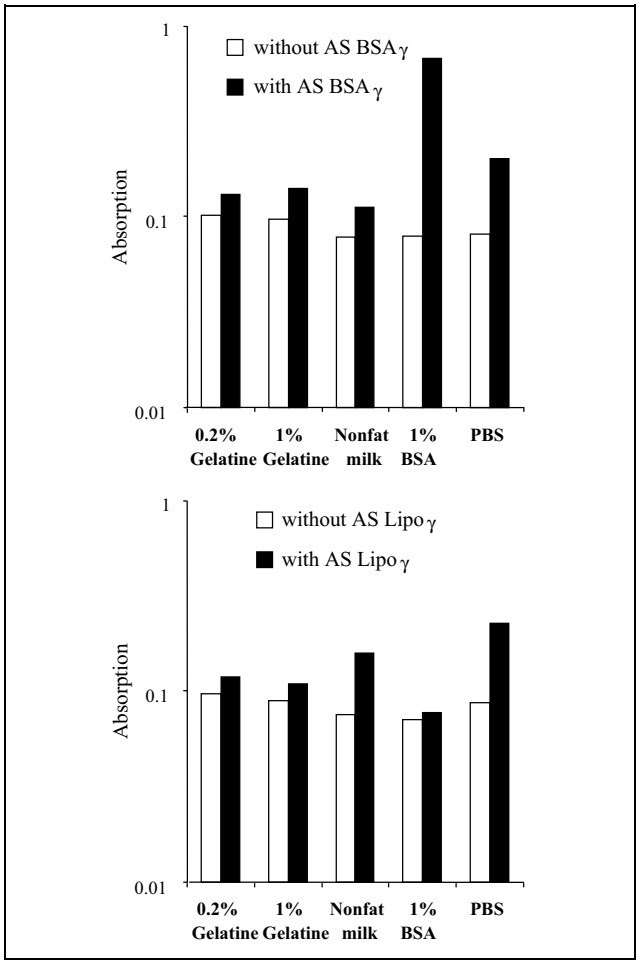


Fig. 3: Cross-reactivity of *AS BSAγ* and *AS lipoγ* with different blocking reagents

blocking step is required. To exclude possible cross-reactivity of the antiserum and blocking reagents, an assays was carried out: Free binding sites on the microwell surface were blocked using different blocking buffers after a comprehensive screening with substances often used in literature had been performed (Fig. 3). In the case of *AS BSAγ,* 0.1% nonfat dried milk in PBS buffer was used, while 1% BSA/PBS buffer was used in the case of *AS lipoγ*.

2.4.3. Screening experiments

After the elimination of unspecific side reactions in the fine tuning of the experiments, several rabbit γ-globulin-fractions were evaluated to determine the fraction with the highest antibody-potency for ELISA. Screening experiments showed that the antigenic potential of the hapten-lipopeptide conjugate was significantly lower when compared to the hapten-BSA conjugates, even though the immunization period of the hapten-lipopeptide conjugate was twice as long (Fig. 4). Thus, the ELISA was developed with an antiserum that resulted from BSA-hapten conjugate.

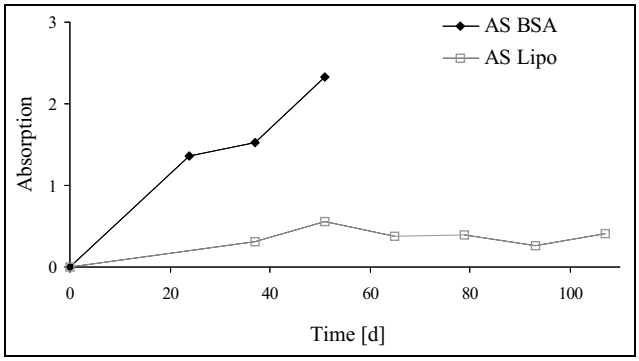


Fig. 4: Comparison between the antibody titers of *AS BSA* and *AS lipo* by measurement of test bleeds taken during the immunization period

2.4.4. Validation

The validation of the optimized ELISA was performed with respect to the limits of quantification, precision and accuracy, as well as the influence of a human urine matrix. The total error for each determination (n = 5) in the investigated optimum concentration range was always less than 10% (Fig. 5). Based on a 90% confidence limit, upper and lower limits of quantification were 362 and 3.62 ng *S*-ibuprofen/ml, respectively (Fig. 6).

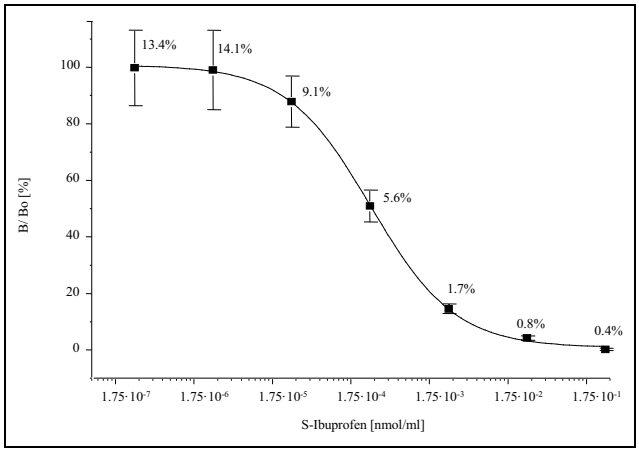


Fig. 5: Total error determination (n = 5) using logistic 4-parameter-adjustment

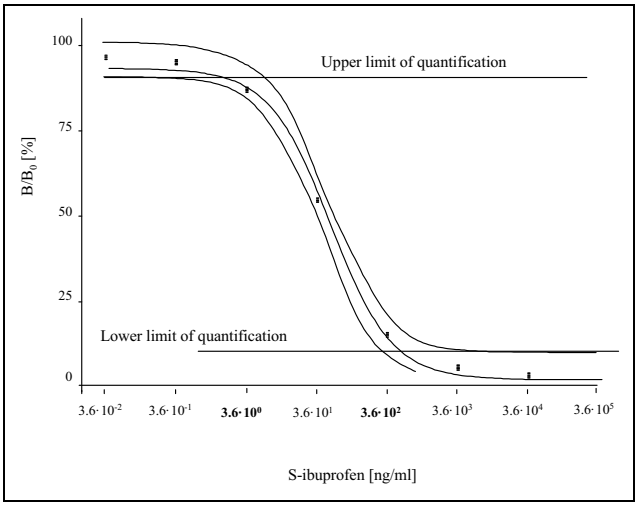


Fig. 6: Upper and lower limits of quantification based on 90% confidence interval

In this concentration range, recovery rates were between 89.8% and 100.5% (11.5 ng/ml, n = 5) and between 99.4% and 102.4% (115 ng/ml). Intra-assay precision (CV%) was 13.2% (11.5 ng/ml, n = 5), and 11.1% (115 ng/ml), inter-assay precision (CV%; 3 test-series; n = 5) was 5.3% and 1.3% (115 ng/ml), respectively. Recovery rates in *S*-ibuprofen-spiked human urine samples (3 test series, n = 5) ranged from 91.2% to 100.5 % (11.5 ng/ml) and from 99.4% to 102.0% (115 ng/ml). Student's t-test showed no statistically significant difference (p = 0.1) between the recovery rates for buffer and spiked urine samples.

2.4.5. Cross-reactivity

Characterization of the polyclonal antiserum in cross-reactivity experiments using compounds with analogous structure demonstrated that the isobutyl group of ibuprofen is highly relevant for its antigenic recognition and that the polyclonal antiserum possesses a great specificity for *S*-ibuprofen (cross-reactivity <0.14%–1.36%, Fig. 7). Hence, in therapeutic drug monitoring (TDM) the developed ELISA allows a quantification of *S*-ibuprofen in the presence of other NSAIDs in the low nanogram-range.
Additional cross-reactivity experiments were carried out to receive detailed information about the antigenic recogni-

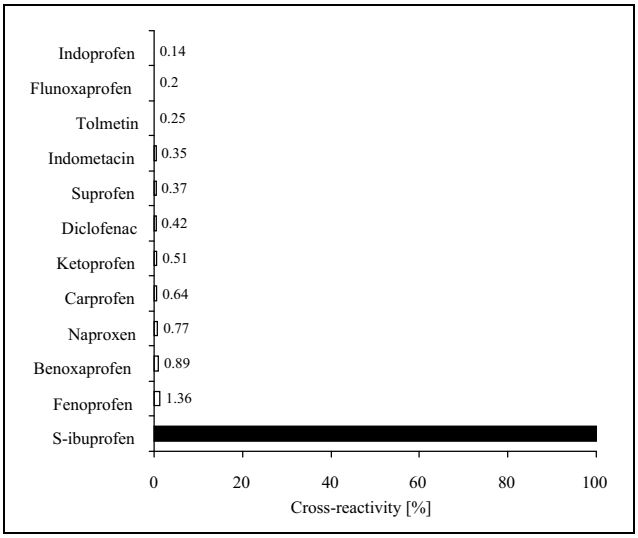


Fig. 7: Cross-reactivity of *AS BSAγ* with various analogous compounds.

tion concerning the chiral center. Therefore, analogous compounds were used, which varied regarding the steoreoisomerism (*R*-ibuprofen), the chiral center (ibufenac), and the isobutyl group (isopropylphenylacetic acid). These experiments demonstrated that there was – in addition to the specific antibody population for *S*-ibuprofen another antibody population, which is expressed by the cross-reactivity of *R*-ibuprofen (50%) and ibufenac (58%). The fact that the cross-reactivity of both compounds is in the same range leads to the suggestion that the antigenic recognition of the additional antibody population contained only the isobutyl and phenyl structure of ibuprofen. This result is caused by the use of a polyclonal antiserum, hence, the use of a monoclonal antiserum should overcome this problem.

The different existing antibody populations in the polyclonal antiserum were elucidated with the help of molecular modeling studies. Figure 8 shows that the conformation of *S*- and *R*-ibuprofen is identical with respect to the isobutyl- and phenyl residue, even though the configuration at the chiral center is different. Moreover, the differences regarding the chiral center are inferior – in both cases the methyl group lies on the same side of the plane. Due to the high conformational similarity and because of the fact that the linkage to BSA was carried out through the carboxyl function of *S*-ibuprofen, the antigenic recognition of *S*-ibuprofen may be impeded. Therefore, an additional antibody population resulted, whose antigenic recognition did not contain the chiral center. In contrast, the different conformations of the isopropyl and isobutyl residues lead to the reduction in the cross-reactivity to 6.4%.

Despite the fact that the antiserum resulting from the hapten-lipopeptide conjugate was less suitable for the estimated quantification range of *S*-ibuprofen due to its weak

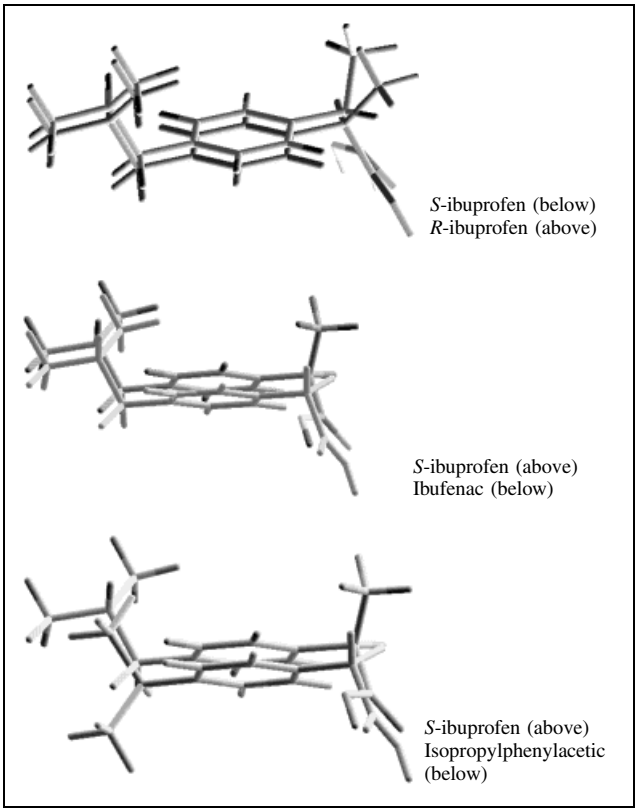


Fig. 8: Molecular modeling of S-ibuprofen (above) and R-ibuprofen (below), S-ibuprofen (above) and ibufenac (below), and S-ibuprofen (above), and isopropylphenylacetic acid (below).

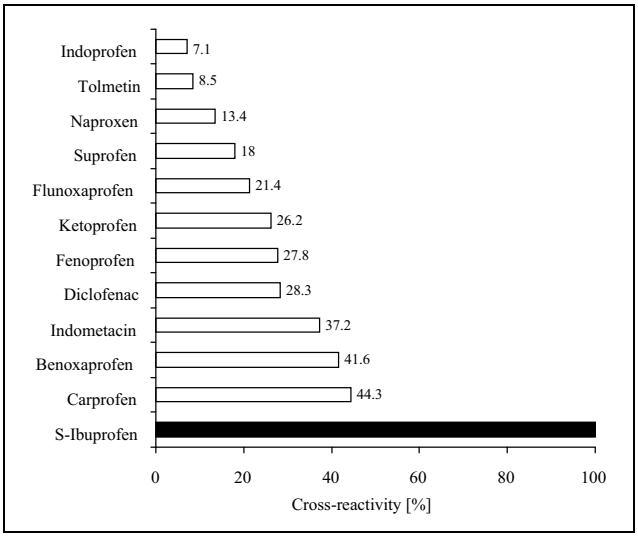


Fig. 9: Cross-reactivity of *AS lipoγ* with various analogous compounds.

antibody potency, an ELISA was developed using this antiserum in order to receive information about the different antibody populations. The characterization experiments with the structural analogues showed a significantly lower specificity for *S*-ibuprofen in this antiserum, which is expressed in the cross-reactivity results (7.1–44.3 %, Fig. 9).

In conclusion, the developed indirect heterogeneous competitive ELISA allows a direct routine determination of *S*-ibuprofen in the presence of other NSAIDs in urine samples in the low nanogram-range without sample preparation and high sample throughput.

3. Experimental

3.1. Materials and methods

S-ibuprofen, 4-aminobutyric acid, *N*-hydroxysuccinimide, dicyclohexylcarbodiimide, and potassium thiocyanate were obtained from Sigma-Aldrich (Deisenhofen, Germany) as well as the immunochemicals anti-rabbit whole serum, anti-rabbit IgG-POD, poly-L-lysine, and 2,2′-azino-di-[3-ethylbenzthiazoline-6-sulfonate] (ABTS). Bovine serum albumin (BSA) fraction V and *S*-[2,3-Bis-(palmitoyloxy-)-(2-*R*,*S*)-propyl]-*N*-palmitoyl-(*R*)-Cys-(*S*)-Ser-(*S*)-Lys_4-OH [(Pam_3Cys-Ser-$(Lys)_4$] were purchased from Boehringer Mannheim (Mannheim, Germany).

^{1}H- and ^{13}C NMR-spectra were recorded with an ARX 300 and an AC 200 E spectrometer (Bruker-Physics, Rheinstetten-Forchheim, Germany), respectively, using tetramethylsilane as internal standard. MS was carried out with a MAT 212 spectrometer (Finnigan MAT, Bremen, Germany) and 0.1 M esculetin as internal standard.

HPLC analyses were performed using a Waters module 510 HPLC pump in combination with a Waters 486 Tunable Absorbance Detector and a HP 3394 A Integrator (Hewlett Packard, Bad Homburg, Germany). For the examination of the purity of *S*-ibuprofen-4-aminobutyric acid-*N*-hydroxysuccinimide and for the determination of the labeling degrees of BSA-hapten-conjugates, a stainless steel column (250 × 4 mm) was used, prepacked with Spherisorb ODS 2.5 µm (Knauer, Berlin, Germany). The mobile phase consisted of methanol/water (70 : 30) at a constant flow rate of 1 ml/min, UV detection was performed at 225 nm. For the examination of the preservation of the *S*-conformation, a chiral acid glycoprotein (AGP) column (100 × 4 mm) was used with 0.01 M monobasic sodium phosphate pH 7.0 (110 µm/l *N*,*N*-dimethyloctylamine were added) as mobile phase, which was delivered at a constant flow rate of 0.9 ml/min.

Tangential flow dialysis of the BSA-hapten conjugates was performed using the tangential-flow Millipore Minitan®-S Ultrafiltration System (Bedford, USA) with a pump head at 480 ml/min. Dialysis was performed using a low Millipore binding cellulose membrane PLGC OMS 10 (MW cutoff 10000).

The dialysis tubing, Visking Type 36/32 (MW cutoff: 12000–14000, flat width 44 mm, wall thickness 25 µm, diameter 27 mm, 6.42 ml cm), which was used for the dialysis of the KSCN-treated BSA-hapten conjugates, was prepared by subsequent washing with purified water.

Lyophilization was performed with an Edwards Modulyo freeze dryer and a high-vacuum pump E2 M8 (Crawley, England).

For the isolation of the γ-globulin fractions, a Pharmacia LKB XK 16 column (1.6 × 40 cm), a flow cell LKB (Bromma) 2238 Uvicord S II), and a Pharmacia LKB Rec 101 were used (Uppsala, Sweden). The mobile phase consisted of 0.03 M sodium phosphate buffer pH 7.0, the solid phase was DEAE cellulose (Whatman, Kent, USA).
Electrophoresis was carried out with a Phero-stab 300 (Biotech-Fischer, GmbH Reiskirchen, Germany), and Tris buffer pH 8.2 (12.1 g Tris-hydroxymethyl-aminomethan and 458 ml 0.1 M HCl dissolved in 2 l water).
The ELISA equipment consisted of a Microtiter-Plate Photometer MR 5000 (Dynatech, Iserlohn, Germany) in combination with a 486-DX40 PC, and the Dynatech Biolinx-software (V. 2.21). Washing of the microtiter plates was carried out with a Dynatech 16-way floating head Multi-Reagent-Washer.
The following buffers were used for the establishment of the ELISA: Coating buffer (0.025 M phosphate buffer pH 7.2), phosphate-buffered saline (PBS) pH 7.2 (0.2 g KH_2PO_4, 2.9 g $Na_2HPO_4 \times 12\ H_2O$, 8.0 g NaCl, and 0.2 g KCl in 1 l water), blocking buffer (1% dried nonfat milk/PBS buffer), washing buffer pH 7.2 (0.1% Tween 20 in PBS), ABTS-buffer pH 4.4 (0.5 g $NaBO_4$, 7.67 g citric acid, and 10.66 g $Na_2HPO_4 \times 2\ H_2O$ in 1 l water), phosphate buffer pH 7.4 for dialysis, and 0.03 M phosphate buffer pH 7.0.

3.2. Syntheses of the BSA-hapten conjugates

3.2.1. Synthesis of S-ibuprofen-4-aminobutyric acid

3.2.1.1. Preparation of activated *S*-ibuprofen

S-Ibuprofen (1.00 g, 4.9 mmol) was suspended in a round-bottom two neck flask in 10 ml toluene, then 3 ml thionyl chloride in 8 ml toluene were added using a dropping funnel, this solution was refluxed for 3.5 h. After cooling the excess of thionyl chloride and toluene was separated using a 12 cm Vigreux distilling column (20 Torr/40 °C). A yellow oily residue resulted.

3.2.1.2. Synthesis of *S*-ibuprofen-4-aminobutyric acid using a modified Schotten-Baumann reaction [19]

4-Aminobutyric acid (0.602 g, 5.84 mmol) and 1.14 g (13.75 mmol) Na_2CO_3 were dissolved in 6 ml H_2O in a round-bottom two neck flask, then 4.4 ml 10% NaOH were added. *S*-Ibuprofen chloride was added to the ice-cooled solution of 4-aminobutyric acid slowly dropwise using a dropping funnel and the mixture was stirred for 4 h. Afterwards, the solution was acidified using 15 ml 9% HCl. The resulting white solution was transferred to a separatory funnel and extracted 3 times with 10 ml CH_2Cl_2. The organic layer was washed with H_2O, dried over Na_2SO_4 and the solvent evaporated. The oily residue was dissolved in 4 ml C_2H_5OH and treated with 10 ml n-hexane and 5 ml H_2O. The residue, recrystallized twice from n-hexane, was pure, with a yield of 88.5% and 1.27 g, respectively.
^{1}H−NMR (300 MHz), ($CDCl_3$) δ (ppm): 0.90 (d, $^3J = 6.53$ Hz, 6 H, $\underline{H}_3C-CH-C\underline{H}_3$), 1.51 (d, $^3J = 7.16$ Hz, 3 H, $\underline{H}_3C-CH$), 1.72–1.79 (m, $^3J = 7.00$ Hz, 2 H, $-CH_2-C\underline{H}_2-CH_2-$), 1.83–1.87 (m, $^3J = 6.79$ Hz, 1 H, $(CH_3)_2-C\underline{H}-CH_2$-), 2.28 (t, $^3J = 7.00$ Hz, 2 H, $-CH_2-CH_2-C\underline{H}_2$-), 2.45 (d, $^3J = 7.17$ Hz, 2 H, $-C\underline{H}_2-CH$), 3.25 (q, $^3J = 6.73$ Hz, 2 H, $-NH-C\underline{H}_2-CH_2-$), 3.54 (q, $^3J = 7.22$ Hz, 1 H, $-C\underline{H}-CH_3$), 5.59 (s breit, 1 H, $-N\underline{H}-CH_2$), 7.10–7.19 (m, $^3J = 6.00$ Hz, 4 H, $\underline{H}$ arom.), 10.4 (s, 1 H, $-O\underline{H}$), −NH−, −OH. ^{13}C NMR (200 MHz), ($CDCl_3$) δ (ppm): 14.3 (C-17, $\underline{C}H_3CH$); 22.4 (C-15, 16, $(\underline{C}H_3)_2CH$); 24.4 (C-14, $CH_2\underline{C}H_2CH_2$); 30.3 (C-13, $(CH_3)_2\underline{C}H$); 36.0 (C-12, $CH_2\underline{C}H_2C$); 45.4 (C-11, $CH\underline{C}H_2C$); 46.4 (C-10, $\underline{C}HCH_3$); 48.6 (C-9, $NH\underline{C}H_2$); 129.2 (C-7.8$_{arom}$); 129.7 (C5.6$_{arom}$); 133.4 (C-4$_{arom}$); 142.2 (C-3$_{arom}$); 171.0 (C-2); 172.6 (C-1$_{prop}$)

3.2.2. Synthesis of S-ibuprofen-4-aminobutyric acid-N-hydroxysuccinimide

S-Ibuprofen-4-aminobutyric acid (1.00 g, 3.43 mmol) and 393 mg (3.42 mmol) *N*-hydroxysuccinimide were dissolved in 10 ml 1,4-dioxane in a 50 ml round-bottom two-neck flask. To this solution, 785 mg (3.77 mmol) of dicyclohexylcarbodiimide, dissolved in 7 ml 1,4 dioxane, were added. The mixture was stirred for 5 h at room temperature and then cooled, resulting in the precipitation of crystalline dicyclohexyl urea. After the solid was filtered off, the solvent was evaporated and 25 ml CH_2Cl_2 were added to the yellow oily residue. The organic layer was washed four times: with 10 ml 0.01 M HCl, with 10 ml H_2O, with 10 ml 10% $NaHCO_3$ and finally again with 10 ml H_2O. Afterwards, the organic layer was dried over Na_2SO_4 and then evaporated. The oily residue was dissolved in 5 ml CH_2Cl_2 and treated with cyclohexane. The residue, recrystallized twice from cyclohexane, was pure; the yield was 68.4% and 0.91 g, respectively.
^{1}H NMR (300 MHz), ($CDCl_3$) δ (ppm): 0.90 (d, $^3J = 6.60$ Hz, 6 H, $\underline{H}_3C-CH-C\underline{H}_3$), 1.50 (d, $^3J = 7.18$ Hz, 3 H, $\underline{H}_3C-CH$), 1.78–1.86 (m, $^3J = 6.77$ Hz, 2 H, $-C\underline{H}_2-CH_2-$), 1.87–1.95 (m, $^3J = 6.77$ Hz, 1H $(CH_3)_2-C\underline{H}-CH_2-$), 2.42 (d, $^3J = 7.64$ Hz, 2 H, $-C\underline{H}_2-CH$), 2.55 (t, $^3J = 7.46$ Hz, 2 H, $-CH_2-CH_2-C\underline{H}_2-$), 2.84 (s, 4 H, $\underline{H}$ succinimide), 3.31 (q, $^3J = 6.77$ Hz, 2 H, $-C\underline{H}_2-CH_2-CH_2-$), 3.53 (q, $^3J = 7.19$ Hz, 1 H, $-C\underline{H}-CH_3$), 5.68 (s breit, 1 H, $-N\underline{H}-CH_2-$), 7.09–7.20 (m, $^3J = 8.05$ Hz, 4 H, $\underline{H}$ arom.), −NH−. ^{13}C-NMR (200 MHz), ($CDCl_3$) δ(ppm): 14.3 (C-21, $\underline{C}HCH_3$); 22.4 (C-19, 20, $(\underline{C}H_3)_2CH$); 24.4 (C-18 $CH_2\underline{C}H_2CH_2$); 25.4 (C-16,17, $\underline{C}H_2\underline{C}H_{2succ}$); 26.3 (C-15, $CH_2\underline{C}H_2C$); 30.3 (C-14, $(CH_3)_2\underline{C}H$); 45.4 (C-13, $CH\underline{C}H_2C$); 46.4 (C-12, $\underline{C}HCH_3$); 48.6 (C-11, $NH\underline{C}H_2$); 129.2 (C-9, 10$_{arom}$); 129.7 (C-7, 8$_{arom}$); 133.4 (C-6$_{arom}$); 142.2 (C-5$_{arom}$); 172.0 (C-3, 4 2C$_{succ}$); 172.6 (C-2$_{amid}$); 174.5 (C-1).

3.3. Synthesis of the BSA-hapten conjugates

3.3.1. Preparation of BSA-S-ibuprofen conjugates

A solution of *S*-ibuprofen-4-aminobutyric acid in 10 ml of dioxane was added with vigorous stirring to a solution of 500 mg bovine serum albumin in 10 ml water. Subsequently, 1 M NaOH was added dropwise, at which the solution became somewhat turbid. After the reaction had proceeded for 30 min, further addition of 1 M NaOH resulted in a clear solution. Stirring was continued for 24 h at room temperature, then the solution was dialyzed by using tangential flow dialysis and lyophilized.

3.3.2. Determination of labeling degrees using HPLC

In the case of the hapten-BSA conjugates, we used an HPLC method after performing total conjugate hydrolysis. BSA-hapten conjugate (10 mg) was dissolved in 2 ml 6 M HCl in a glass tube and the hydrolysis was carried out at 120 °C for 24 h. To 2.0 ml sample, 250 l flurbiprofen (1 mg/ml) were added as an internal standard. Liquid extraction was performed with 5.0 ml n-hexane/diethylether (8 : 2, 30 s vortex mixer, 30 min agitation, centrifugation at 5000 rpm for 10 min). Organic layer (4.0 ml) was transferred into a glass tube and then evaporated. The residue was reconstituted in 1.0 ml mobile phase of which 20 µl were injected.

3.3.3. Treatment of the conjugates with chaotropic substances

Conjugate (30 mg) was dissolved in 3 ml phosphate buffer pH 7.4 and treated with 3 ml 6 M KSCN. The solution was stirred for 3 h at room temperature and then dialyzed against phosphate buffer pH 7.4. The completeness of the dialysis was indicated using $FeCl_3$. The residue was lyophilized and the characterization was carried out as described above (3.3.2.).

3.4. Synthesis of the lipopeptide-hapten conjugate

3.4.1. Synthesis of S-ibuprofen succinimide

S-Ibuprofen (2.06 g, 10 mmol) were dissolved in 5 ml dioxane in a 100 ml round-bottom flask; 1.15 g (10 mmol) *N*-hydroxysuccinimide in 15 ml dioxane and 2.27 g (11 mmol) dicyclohexylcarbodiimide in 25 ml dioxane were added. After 5 min, dicyclohexyl urea solidified. Stirring was continued for 3 h at room temperature. Then, the solid was filtered off and the solvent was evaporated and CH_2Cl_2/petrolether was added to the residue giving a crystalline mass and yield of 2.31 g and 76.2%, respectively.
^{1}H-NMR (300 MHz), ($CDCl_3$) δ (ppm): 0.90 (d, $^3J = 6.56$ Hz, 6 H, $\underline{H}_3C-CH-C\underline{H}_3$), 1.63 (d, $^3J = 7.17$ Hz, $C\underline{H}_3-CH$), 1.83–1.90 (m, 1 H, $H_2C-C\underline{H}-(CH_3)_2$), 2.46 (d, $^3J = 7.18$ Hz, $C\underline{H}_2-CH-(CH_3)_2$), 2.81 (s, 4 H, $\underline{H}$ succinimide), 4.03 (q, $^3J = 7.16$ Hz, $-C\underline{H}-CH_3$), 7.13–7.27 (m, $^3J = 8.14$ Hz, 4 H, $\underline{H}$ arom.) ^{13}C NMR (200 MHz), ($CDCl_3$) δ (ppm) = 17.0 (C-17, $\underline{C}H_3CH$); 22.4 (C-15, 16, $(\underline{C}H_3)_2CH$); 25.4 (C-13,14, $\underline{C}H_2\underline{C}H_2$); 30.3 (C-12, $\underline{C}H(CH_3)_2$); 42.5 (C-11, $\underline{C}H(CH_3)$; 45.4 (C-10, $CH\underline{C}H_2$); 129.2 (C-8,9$_{arom}$); 129.7 (C-6, 7$_{arom}$); 137.2 (C-5$_{arom}$); 142.2 (C-4$_{arom}$); 172.0 (C-2, 3$_{succ}$); 175.4 (C-1$_{prop}$).

3.4.2. Synthesis of the lipopeptide-S-ibuprofen conjugate

The synthesis was performed with the lipopeptide *S*-[2,3-Bis(palmitoyloxy)-(2-*RS*-)-propyl]-*N*-palmitoyl-(*R*)-Cys-(*S*)-Ser-(*S*)-Lys$_4$-OH × 3 HCl (Pam_3Cys-Ser-Lys$_4$-OH), which provides four amino groups for linking *S*-ibuprofen succinimide via its carboxyl group. A solution of 33.9 mg (111.6 µmol) *S*-ibuprofen succinimide in 2 ml dioxane was added to a solution of 15 mg (9.3 µmol) Pam_3Cys-Ser-(Lys)$_4$-OH 3 HCl in 2 ml of H_2O. After addition of 19 l (148.8 µmol) *N*-ethylmorpholine, the mixture was stirred for 144 h at room temperature. The product solidified on cooling and was gained by centrifugation. The precipitate was washed with a cold mixture of dioxane/water (1 : 1) and lyophilized from *tert*.-butanol. The product was characterized by ^{1}H NMR and Matrix Assisted Laser Desorption Ionization spectrometry.

3.5. Poly-L-lysine-hapten conjugate

To a solution of 20 mg (0.165 µmol 95 µmol lysine monomer) poly-L-lysine in 5 ml dioxane/H_2O (1 : 1), a solution of 28.8 mg (95 µmol) *S*-ibuprofen succinimide in 5 ml dioxane/water (1 : 1) was added. After addition of 26 µl (192 µmol) *N*-ethylmorpholine, the mixture was stirred for 24 h at room temperature, then dialyzed against water and lyophilized. Due to the principle of an indirect ELISA, a separation of unbound *S*-ibuprofen was not necessary.

3.6. Immunization

The immunization was kindly performed by Behring (Marburg, Germany). In the case of the BSA-hapten conjugate, rabbits were treated with 750 μg conjugate dissolved in 1 ml NaCl and *Freund's* adjuvant. Booster injections were carried out on the 15th and 25th day. In the case of the lipopeptide-hapten conjugate, rabbits were treated with 100 μg conjugate dissolved in 1 ml NaCl and *Freund's* adjuvant. Booster injections were carried out on the 16th (200 μg), 42nd (100 μg), 56th (300 μg), and 84th day (300 μg). At the end of each immunization regime, the animals were bled and the γ-globulin fractions were isolated.

3.7. Isolation of the globulin fractions

Native antiserum (10 ml) was treated with 10 mg BSA and stirred for 1 h at room temperature. The solution was clarified by centrifugation and dialyzed against 3 × 1 l 0.03 M phosphate buffer pH 7.0 for 24 h. Because of the gaining volume, the antiserum was concentrated by centrifugation at 1,500 × g for 30 min using a Centriprep 30 (Amicon, Massachusetts, USA). Ion-exchange chromatography was carried out with DEAE Cellulose, then 50 ml DEAE cellulose was added per g protein. For equilibration, DEAE cellulose was washed with 2 l 0.03 M phosphate buffer pH 7.0 using a buchner funnel. Equilibrated antiserum was applied to a DEAE column, IgG-containing fractions were collected and stored at −20 °C.

3.8. ELISA

Coating (200 μl, 4 °C, 24 h)
Poly-L-lysine-*S*-ibuprofen conjugate 6.4 μg/ml in 0.025 M phosphate buffer pH 7.2

Washing 4 × 300 μl washing buffer

Blocking (250 μl blocking buffer, 1 h, room temperature)

Washing 4 × 300 μl washing buffer

Competition step (4 °C, 23 h)
100 μl Sample or 100 μl blank in 1% nonfat dried milk/PBS
100 μl Antiserum (3.13 μg protein/ml 1% nonfat dried milk/PBS)

Washing 4 × 300 μl washing buffer

Addition of secondary antibody (200 μl, 2 h, room temperature)
Anti-rabbit-IgG-POD in 1% nonfat dired milk/PBS

Washing 4 × 300 μl washing buffer

ABTS (200 μl, incubation time 30 min)
Detection 405/490 nm

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