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EVALUATION OF THE EFFICIENCY OF THREE DIFFERENT FLUORESCENT CONJUGATES USED AS TRACERSIN PENICILLIN MAGNETIC NANOPARTICLE IMMUNOASSAY

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Abstract: Three conjugates between the investigated antibiotic Penicillin G (PEN) and fluorescent dyes (ATTO derivatives) were prepared and used as tracers. The first step in all conjugation reactions was activation of PEN by carbodiimide method. Conjugate 1 was synthesized in two steps: activated PEN was conjugated to bovine serum albumin (BSA), and the purified and lyophilized PEN-BSA conjugate was labeled with ATTO 647 NHS dye (PEN–BSA-ATTO 647 NHS conjugate). Conjugate 2 was prepared by using ethylenediamine as a spacer between activated PEN and ATTO 647 NHS (PEN–EDA-ATTO 647 NHS conjugate). Conjugate 3 was synthesized by direct coupling of activated PEN to ATTO 488 amine (PEN–ATTO 488 amine conjugate). The fluorescent conjugates were purified by gel chromatography. A fluorescent immunoassay for PEN determination on the basis of the obtained three conjugates and immobilized anti-penicillin antibody on magnetic nanoparticles (MNPs) was developed. An antibody against PEN was immobilized on MNPs by the random and oriented method. The measuring protocol was based on the principles of a non-competitive indirect fluorescent immunoassay. It was found that the assay sensitivity was the highest when using oriented antibody immobilization and Conjugate 3 (PEN-ATTO 488) as a tracer.

INTRODUCTION

The beta-lactam group is one of the most important families of antibiotic used in veterinary medicine in the treatment of different infections. According to EEC Regulation (Commission Regulation № 508/99, 1999) maximum residue limits (MRLs) have been established in the European Union, currently including seven penicillins, i.e. penicillin G, ampicillin, amoxicillin (4 ng/ml), and oxacillin,

cloxacillin, dicloxacillin and nafcillin, respectively (30 ng/ml). Other countries have similar regulations (MacNeil and Ellis, 1995). Microbiological test systems offer sufficient sensitivity for the detection of penicillins, but also give dose-dependent and variable positive results with other groups of antibiotics. Qualitative penicillin receptor-type assays are increasingly used in industry, but these do not differentiate between penicillins and cephalosporins, and offer no means of quantification (Kroll et al., 2000). Physicochemical methods for the detection of penicillins, such as liquid chromatography/mass spectrometry (Riediker et al., 2001; Holstege et al., 2002), are costly, require extensive sample preparation and clean up procedures prior to analysis and therefore are not suitable for high-throughput routine analysis. The immunoassays now play a major role in veterinary drug residue testing. The advantages of immunoassays techniques compared to other methods include their reliability, speed of analysis, ease of use, little sample preparation, selectivity and sensitivity. Immunoassays with fluorescent detection have the largest application, as the fluorescent labels have several advantages: the amplification of the signal, higher sensitivity detection of the analyte, simplified reagents and simpler assay designs. This study presents a rapid, sensitive and quantitative immunofluorescent method for detection of PEN. The choice of suitable fluorescent conjugates of PEN is a very important factor for sensitivity of the PEN immunoassay.

The objectives of this study were to prepare three fluorescent conjugates of PEN and to investigate their sensitivity as tracers for the development of an immunoassay for PEN. The immunoassay was performed on the basis of immobilized antibody on MNPs. Random and oriented antibody immobilization methods were studied and the obtained analyses were compared.

MATERIALS AND METHODS

Reagents and chemicals

Penicillin G sodium salt (PEN), 98%; N-(3-Dimethylaminopropyl)-N-ethyl-carbodiimide hydrochloride (EDC), 98%; ATTO 488 amine and ATTO 647 NHS; dimethylformamide (DMF); Glutaraldehyde (GA); Ethylene diamine (EDA); Bovine serum albumin (BSA); Tween 80; Sephadex G-10 and G-50. All reagents are purchased from Sigma-Aldrich; anti – penicillin (Ab), 2.2 mg/mL from the Diagnostic Consulting Network; Protein A (PA) from the Diagnostic Consulting Network; phosphate buffer solutions (PB) with pH 7.4 (assay buffer), and pH 6.0; citrate buffer solution (CB) with pH 5.0. All solutions were prepared using deionized water from PURELAB Ultra – system (ELGA, England). All reagents were of analytical grade.

Instrumentation

The UV-Vis spectra of PEN, BSA, ATTO dyes and the obtained conjugates were recorded by Spectrophotometer 6800 (Jenway, England) in the wavelength interval from 200 to 700 nm.

The fluorescence intensity was detected by Cary Eclipse fluorescence spectrophotometer (Varian, USA) at excitation/emission of 501/523 nm for PEN-ATTO 488, 645/669 nm for PEN–EDA-ATTO 647 NHS and PEN–BSA-ATTO 647 NHS conjugates. The spectrophotometer was supplied with temperature controller, which ensured the same temperature of all samples.

METHODS

Syntheses of PEN–BSA-ATTO 647 NHS conjugate

For PEN activation, 0.007 g EDC were added to 1 ml PEN solution (20 mg in 2 ml of 0.1M PB pH 5.6) and mixed for 30 min. Following the protocol described by Imgen BioSciences, Inc., 20 mg BSA were slowly added to this solution, and the reaction mixture was incubated for 1 h at RT and 18 h at 4°C. The obtained conjugate was purified by gel filtration through a Sephadex G-50 column to discard the unreacted reagents and stored at -18°C in lyophilized form. Then, 25 mg of lyophilized PEN-BSA conjugate were dissolved in 0.9 ml of 0.1M PB pH 8.0 and mixed with ATTO 647 NHS (0.5 mg in 0.1 ml DMF), according to Sigma-Aldrich protocol №1. The labeling reaction was carried out for 2 h at RT and in brown vial to prevent photo degradation of the fluorescent compound. The final fluorescent conjugate was purified by gel filtration through Sephadex G-50 column and lyophilized and stored at -18°C.

Syntheses of PEN–EDA-ATTO 647 NHS conjugate

0.0043 g PEN and 0.023 g EDC were dissolved in 0.2 ml of 0.1M PB pH 5.6 and mixed for 30 min. 0.1 ml of 100 % EDA was dissolved in 0.9 ml dH_2O, following the procedure described by Chu et al. pH of EDA solution was adjusted to 8.5 with the addition of approximately 0.2 ml of 12N HCl. This solution was slowly added to the activated PEN solution and incubated for 2.5 h at RT and continuous shaking and kept 18 h at 4°C. Then, 0.5 mg ATTO 647 NHS dissolved in 50 µl DMF were added to the PEN-EDA conjugate and incubated for 2 h, according to Sigma-Aldrich labeling protocol №1. The final PEN-EDA-ATTO 647 conjugate was separated from unreacted reagents by gel filtration through Sephadex G-10 column and lyophilized and stored at -18°C.

Syntheses of PEN–ATTO 488 amine conjugate

0.002 g PEN and 0.019 g EDC were dissolved in 0.5 ml of 0.1M PB pH 5.6 and mixed for 30 min. Following the Sigma-Aldrich labeling protocol №2, 0.5 mg ATTO 488 amine were dissolved in 50 µl DMF and added to activated PEN solution. The reaction was carried out for 1 h at room temperature (RT) and 18 h at 4°C. The PEN-ATTO 488 conjugate was purified by gel filtration through Sephadex G-10 column and lyophilized by freeze dryer (VirTis, USA) and stored at -18°C.

Glutaraldehyde activation of magnetic nanoparticles (MNPs)

The MNPs were dispersed by sonication for 10 min. A volume of 200 µl MNPs (25 mg/ml) was transferred into 1.5 ml microcentrofuge tube containing 1ml 5% GA in 10mM PBS (pH 8.0) and left for incubation for 4 h at RT with

orbital agitation (250 rpm). The MNPs were washed three times with PB buffer and suspended in 1 ml of the assay buffer for random antibody immobilization or in 1 ml of 10mM CB pH 5.0 for oriented antibody immobilization.

Random antibody immobilization (RI) on the activated MNPs

After the activation step, 10 µl of antibody (2.2 mg/ml) was added to MNPs (5 mg/ml) and incubated for 2 h at 300 rpm and RT. The MNPs-Ab was collected with a magnet, washed three times and the supernatant was used for the unbound antibody determination. 1 ml of 0.5% BSA and 0.05% Tween 80 in 10 mM PB pH 7.4 (blocking buffer) was added to block the unreacted active amine groups for 1 h at RT. The MNPs-mAb were washed four times with the assay buffer and suspended in 1 ml of the same buffer to reach a 5 mg/ml stock solution.

Oriented antibody immobilization (OI) on the activated MNPs

After the activation step, 0.6 mg PA were added to 1 ml of MNPs (5 mg/ml) and incubated for 30 min at RT. The MNPs-PA was collected with a magnet, washed three times and the supernatant was used for the unbound antibody determination. 1 ml of 0.5% BSA and 0.05% Tween 80 in 10 mM CB pH 5.0 (blocking buffer) was added to block the unreacted active amine groups for 1 h. Then, the MNPs were washed four times with the assay buffer and suspended in 1 ml of the same buffer. An antibody, 10 µl, was added to the MNPs-PA and incubated for 2 h. The MNPs-PA-Ab were collected with a magnet, washed three times with the assay buffer and suspended in 1 ml of the same buffer to reach a 5 mg/ml stock solution.

Fluorescent immunoassay procedure

In a first step, the MNPs (75 µl, 5 mg/ml) with immobilized antibody were mixed with an unlabeled antigen (50 µl of 0÷50 ng/ml of PEN standards) and further incubate for 15 min at 37 °C with continuous shaking. The tracer, 100 µl of PEN–ATTO 488, PEN-EDA-ATTO 647 and PEN-BSA-ATTO 647 with a concentration of 400 ng/ml, 1000 ng/ml and 68 µg/ml, respectively, was added and the mixture was pre-incubated for 15 min to allow binding of the tracer to the available antigen binding sites. After incubation, the MNPs complex was separated from the supernatant on the tube side wall by a magnetic separator. The clear supernatant, containing unbound tracer was separated and adjusted to 0.5 ml with the assay buffer. The fluorescence signals of the free, unbound tracer were inversely proportional to the concentration of the unlabeled antibiotic in the samples.

Experimental signals were normalized using the following expression:

$$\textbf{Normalized signal} = (B_0 - B)/(B_0 - Bx).\ 100,\ \%$$

where: B_0 is the fluorescence signal of initial tracer solution; **B** is the fluorescence signal measured in the presence of analyte; **Bx** is the fluorescence signal in the absence of analyte (0 ng/ml).

RESULTS AND DISCUSSION

In this paper, three conjugates with PEN and ATTO fluorescent dyes were prepared in three different methods. Figure 1 shows the scheme for preparation of the three conjugates.

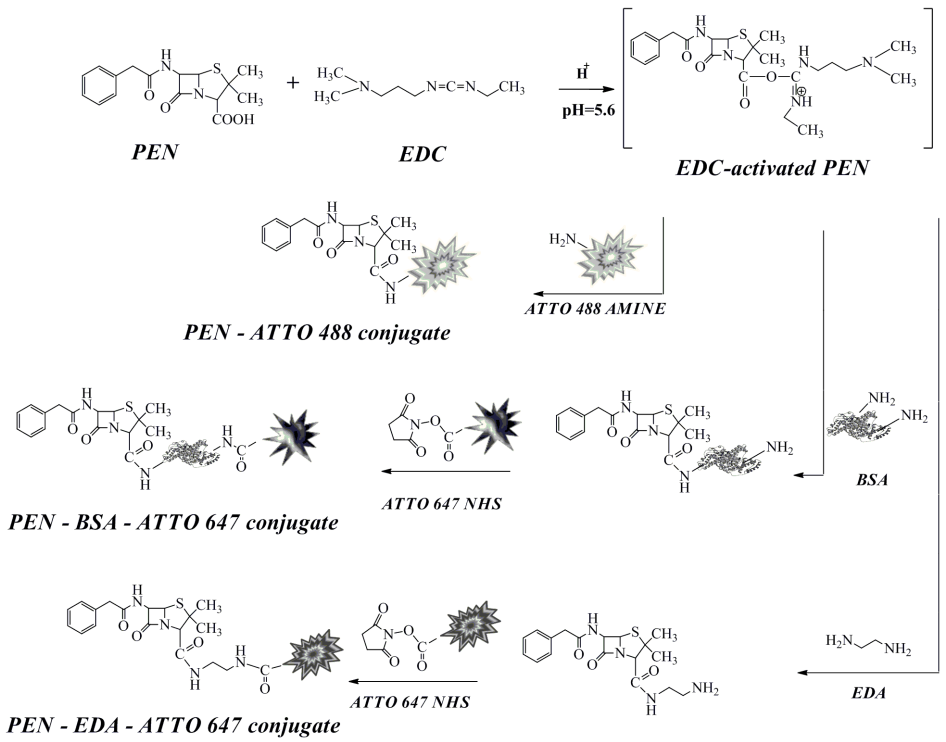

Figure 1. Reaction scheme for preparation of PEN-EDA-ATTO 647, PEN-BSA-ATTO 647 and PEN-ATTO 488 conjugates.

The first step in all conjugation reactions was activation of the PEN carboxyl group by carbodiimide method which is one of the most widely used conjugation methods. The carbodiimide first reacted with available carboxylic groups on PEN to form a highly reactive O-acylisourea intermediate. The activated carboxylic group then reacted with a primary amine to form an amide bond, with the release of the EDC mediator as a soluble isourea derivative. pH near 6 was chosen for carrying out the activation. Conjugate 1 (PEN-BSA-ATTO 647) was prepared in two steps. First, PEN-BSA conjugate was obtained by coupling PEN carboxyl group and BSA amine group and further labeled with fluorescent dye. For preparation of Conjugate 2 (PEN-EDA-ATTO 647), ethylenediamine was used as a spacer between activated PEN and ATTO 647 NHS. First, one of the amine groups of the bifunctional reagent ethylenediamine was coupled with the

PEN carboxyl group. Then, the other amino group was "attacked" by the highly reactive toward amines NHS group of fluorescent dye to form a stable amide bond. Conjugate 3 (PEN-ATTO 488) was synthesized by direct coupling of an activated carboxyl group of PEN to amine group of the fluorescent tracer. The result was the formation of the amide bond.

A purification step was needed to separate the conjugates from the excess of reagents and lateral products after the conjugation. Considering the differences in the molecular weight of the individual components and of the conjugate, purification of all conjugates was carried out by gel filtration chromatography. Sephadex was used as a stationary phase for gel filtration chromatography. The column flow rate was 1 ml/min and 1 ml fractions were collected. Then, the absorbance value of each fraction at different wavelengths (where individual components absorbed) was plotted versus fraction number for each conjugate. The results from column separation of the three conjugates are presented as inserted graphics in Figures 2, 3, 4 and 5.

To obtain evidence of successful conjugation, UV-Vis absorbance of all conjugates was measured and compared to UV-Vis absorbance of the individual components. All reagents were freshly dissolved in 2 ml solvent and the absorbance was measured at wavelength slit of 5 nm and scanning rate of 300 nm/min. The results are shown in Figures 2, 3, 4 and 5.

Conjugate 1 was prepared in two steps – PEN-BSA conjugate was obtained in a first step and further labeled with ATTO dye. Figure 2 shows a comparison between UV-Vis spectra of PEN-BSA conjugate and PEN and BSA spectra in the unconjugated form. As can be seen, BSA and the conjugate had characteristic peaks at 278 nm and 273 nm, respectively. Inserted graphic show conjugate content in fractions from 2 to 7 and an excess of unreacted BSA in fractions 11÷14.

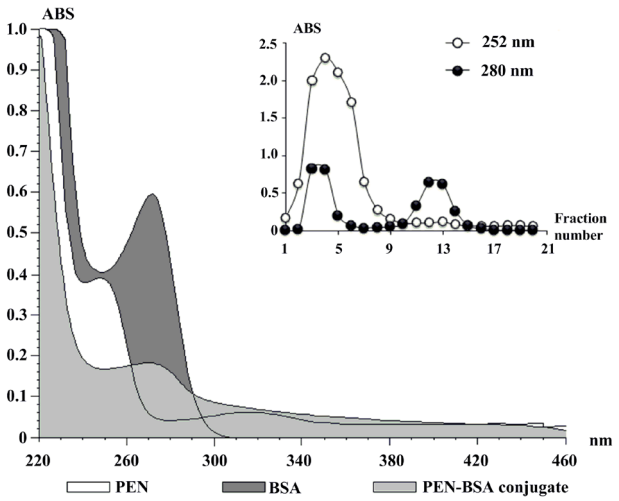

Figure 2. UV-Vis absorption spectra of PEN, BSA and PEN-BSA conjugate.

Figure 3 inserted graphic show 3 peaks. First peak (fractions 1÷4) represented the PEN-BSA-ATTO conjugate, second peak – the unreacted PEN-BSA conjugate, and the third peak represented excess of unreacted ATTO dye. Comparison of the UV-Vis spectrum of the these compounds show slight shifting of the conjugate peak from 645 to 642 nm.

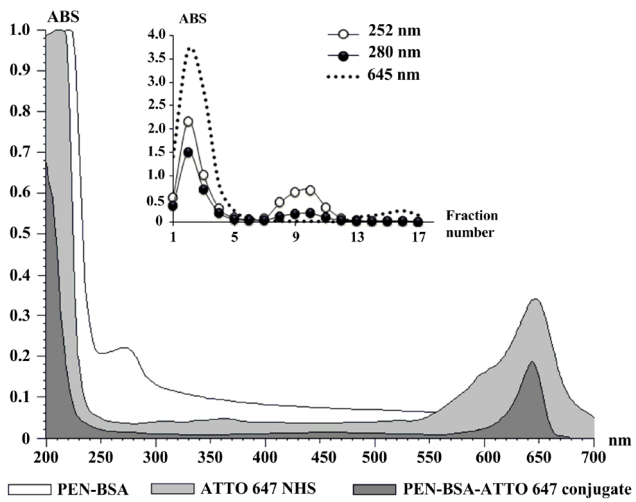

Figure 3. UV-Vis absorption spectra of PEN-BSA, ATTO 647 NHS and PEN-BSA-ATTO 647 conjugate.

Figure 4 shows UV-Vis spectrum of PEN, ATTO 647 and the obtained Conjugate 2, recorded from 200 nm to 700 nm. It was observed that unconjugated ATTO 647 had one peak at 645 nm while the conjugate had two peaks at 275 and 643 nm. UV-Vis absorbance of PEN-EDA-ATTO 488 was shifted from 645 to 643 nm. In inserted graphic, first peak (fractions 1÷10) represented the obtained conjugate, and fractions from 16 to 19 represented free, unbound PEN.

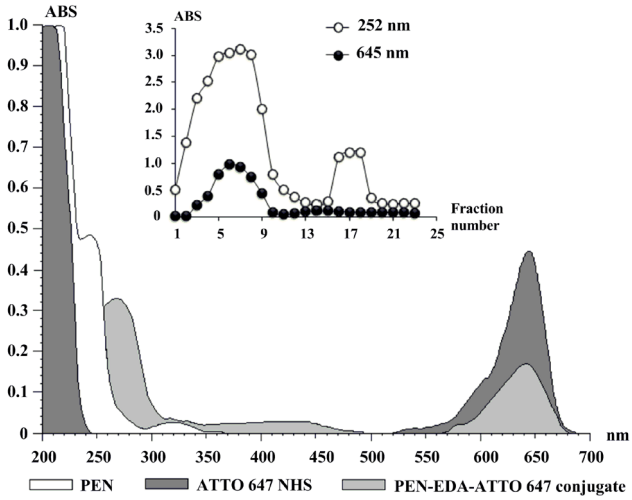

Figure 4. UV-Vis absorption spectra of PEN, ATTO 647 NHS and PEN-EDA-ATTO 647 conjugate.

Figure 5 shows UV-Vis spectrum of PEN, ATTO 488 and the obtained Conjugate 3 recorded from 200 nm to 550 nm. It was observed that PEN had two peaks at 250 nm and 322 nm; ATTO 488 dye had two small peaks at 240 and 305 nm and one major peak at 501 nm while the conjugate had only one peak at 500 nm. Conjugation between PEN and ATTO 488 had slightly shifted the reading of UV-Vis absorbance from 501 to 500 nm. In inserted graphic, first peak (fractions 1÷6) containing both components simultaneously and correspond to the obtained conjugate, and fractions from 10 to 15 correspond to free, unbound PEN.

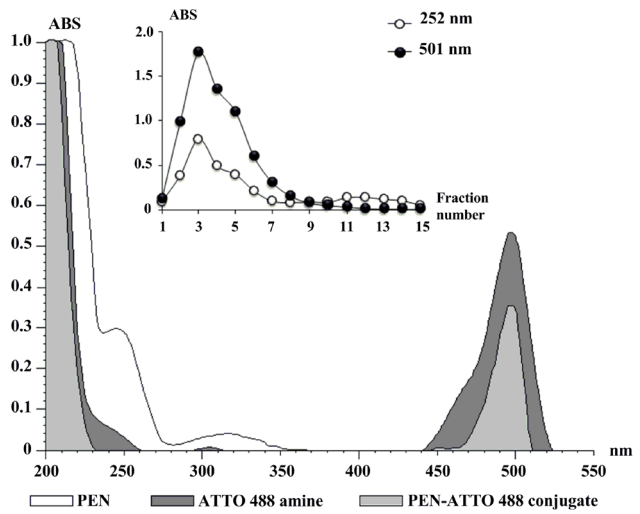

Figure 5. UV-Vis absorption spectra of PEN, ATTO 488 and PEN-ATTO 488 conjugate.

The results of these UV-Vis spectra revealed that PEN was successfully conjugated with fluorescent dyes in all three methods. The conjugates were used as tracers in PEN fluorescent immunoassay and parameters such as the best possible sensitivity, stability and economy were studied.

For development of the assay, anti-penicillin antibody was covalently coupled through their amino groups to the MNPs functionalized with amino groups. Two different strategies of antibody immobilization - oriented and random immobilization on MNPs surface were investigated. When antibodies are covalently attached to solid supports, their specific binding capacity is usually less than that of soluble antibodies. One of the main reasons for this reduction is attributed to the random orientation of the antibodies on support surfaces. This may result in different orientations of the antibody on the support surface depending on which lysine group binds to the support - fully active, partially active or inactive antibody (Figure 6a, random immobilization). When immobilization occurs through the antigen binding sites on the Fab' portions, the ability of that antibody to bind antigen may be severely impaired or eliminated entirely. To eliminate these disadvantages, antibody was attached to MNPs via protein A (oriented immobilization). Schematic diagram of the principles of the fluorescent MNPs immunoassay of random and oriented immobilization method is shown in Figure 6 b).

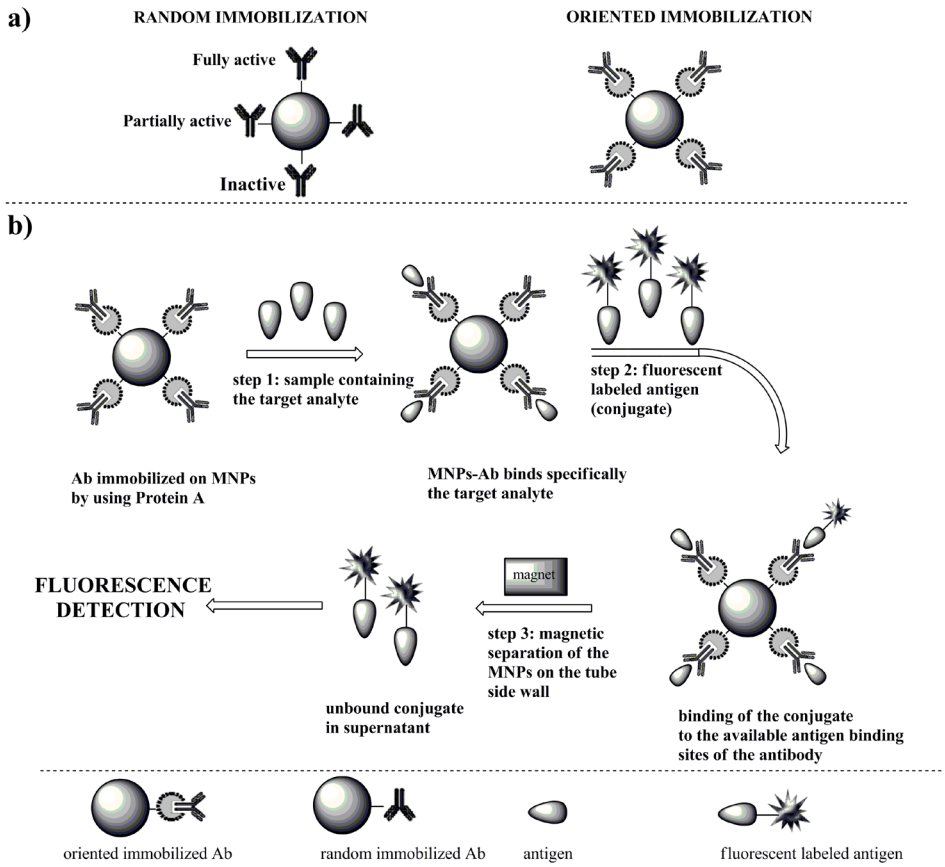

Figure 6. a) RI and OI antibody immobilization method;
b) Principles of the fluorescent MNPs immunoassay by oriented immobilization method.

The fluorescent procedure was based on the principles of non-competitive indirect fluorescent immunoassay. In a first step, the immobilized antibody bound specifically the target analyte (unlabeled antigen PEN) in the sample. In a second step, fluorescently labeled antigen (PEN-BSA-ATTO 647, PEN-EDA-ATTO 647 or PEN–ATTO 488,) bound to the available antigen binding sites of the antibody. After magnetic separation of the MNPs on the tube side wall, fluorescent intensity of the free, unbound tracer in the supernatant was measured. The signal was inversely proportional to the concentration of the unlabeled antigen in the samples.

The efficiency of three conjugates as a tracer in PEN immunoassay was evaluated. Two analyses, using random and oriented immobilization of polyclonal anti–penicillin on MNPs, were carried out and compared. The results are presented in Figure 7.

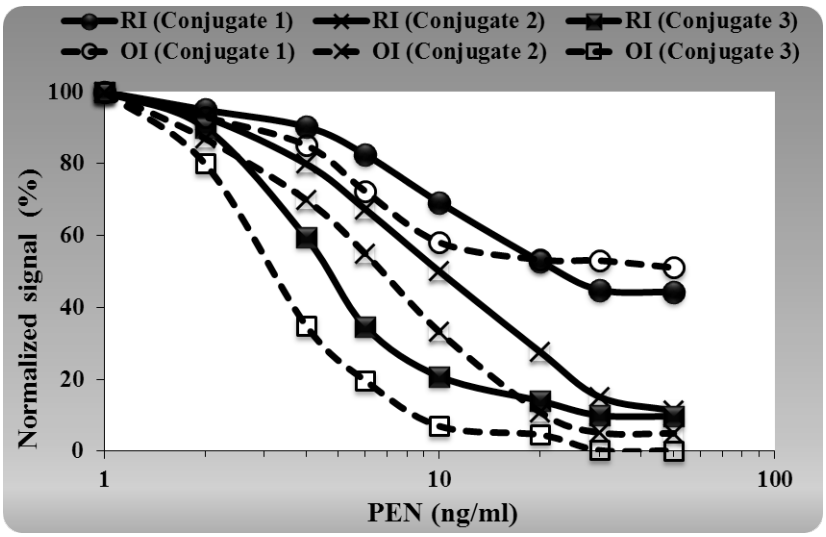

Figure 7. Comparison of PEN calibration curves in PB buffer pH 6.0 using random (solid line) and oriented (dashed line) immobilization of antibody on MNPs and three different fluorescent conjugates as tracers: PEN-BSA-ATTO 647 (Conjugate 1); PEN-EDA-ATTO 647 (Conjugate 2) and PEN-ATTO 488 (Conjugate 3).

The basic characteristics of immunoassay (linear range of PEN concentration, limit of detection – LOD, and IC_{50}) were presented in Table 1. IC_{50} is the concentration of antigen giving 50% inhibition of initial normalized signal. LOD was calculated as 95% normalized signal. The immunoassay sensitivity is usually expressed in terms of its lower LOD, lower IC_{50} and higher slope of the curve. First, the efficiency of PEN-BSA-ATTO 647 conjugate 1 as a tracer in PEN immunoassay was evaluated. The results show that IC_{50} by RI method was 23.4 ng/ml, while IC_{50} by OI method was not determined. Moreover, the slope of the OI curve was slightly higher than the RI curve. The advantage of using OI method was the lower LOD achieved – 2.41 ng/ml in comparison to 3.33 ng/ml by RI method. Also, the linear range using RI method was from 4 to 30 ng/ml, and by using OI method – from 4 to 10 ng/ml.

Table 1. The linear equations and correlation coefficients of PEN calibrations curves using different immobilization methods and different conjugates.

Conjugate type *	Method type**	Linear range, ng/ml	Linear equation	R^2	LOD, ng/ml	IC_{50}, ng/ml
Conjugate 1	RI	4-30	y= -23.1ln(x)+122.85	0.998	3.33	23.42
	OI	4-10	y= -24.99ln(x)+116.99	0.986	2.41	-
Conjugate 2	RI	4-20	y= -32.71ln(x)+125.58	0.999	2.55	10
	OI	2-20	y= -34.08ln(x)+113.72	0.991	1.74	6.49
Conjugate 3	RI	2-10	y= -44.35ln(x)+119.73	0.984	1.75	4.82
	OI	2-10	y= -45.71ln(x)+105.97	0.951	1.27	3.40

*Conjugate type: Conjugate 1 is **PEN-BSA-ATTO 647**; Conjugate 2 is **PEN-EDA-ATTO 647**; Conjugate 3 is **PEN-ATTO 488**;
**Method type - type of antibody immobilization method: RI-random immobilization; OI-oriented immobilization.

These results were unsatisfactory and there was need to improve the assay sensitivity. One of the possible solutions was to prepare a new conjugate, providing better sensitivity. For the second conjugate preparation, EDA was used as a spacer between PEN and ATTO 647, while in PEN-BSA-ATTO 647 preparation, BSA was used as a linker between the antibiotic and the fluorescent dye. The smaller molecule of EDA was chosen to improve the conjugate flexibility and also to reduce steric hindrance of the molecules. The results of the PEN determination by RI and OI methods and PEN-EDA-ATTO 647 conjugate 2 has also been presented in Figure 7. As can be seen, all of the immunoassay parameters were improved (Table 1). The linear ranges of RI and OI curves when using PEN-EDA-ATTO 647 as a tracer was from 4 to 20 ng/ml and from 2 to 20 ng/ml, respectively. LOD by RI method was 2.55 ng/ml and by OI method-1.74 ng/ml, which increased the assay sensitivity to 23 and 28%, respectively, in comparison to PEN-BSA-ATTO 647 assay. Also, IC_{50} decreased to 10 and 6,5 ng/ml using RI method and OI method respectively. The obtained results show that the second conjugate, PEN-EDA-ATTO 647, was more suitable and determined very low concentrations of PEN. However, the initial point of the linear range was above the maximum residue limits for PEN (4 ng/ml).

Therefore, a third conjugate PEN-ATTO 488 between the antibiotic and a new fluorescent tracer was prepared without using any spacers. The free amine group of ATTO 488 dye was directly coupled to the PEN carboxyl group to obtain PEN-ATTO 488 conjugate. The results of the PEN determination with PEN-ATTO 488 conjugate are shown in Figure 7. This tracer provided the lowest IC_{50} and LOD values, and also the highest sensitivity. The slopes of the PEN calibration curve were the highest (44.35 and 45.71 for RI and OI methods, respectively). Also, both RI and OI methods allowed determination of PEN in concentrations below maximum residue limits. It was found that the assay sensitivity was the highest when using oriented antibody immobilization and Conjugate 3 (PEN-ATTO 488) as a tracer.

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