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COMPARISON OF FOUR DIFFERENT METHODS FOR DEVELOPMENT OF PROGESTERONE FLUORESCENT IMMUNOASSAY

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Abstract: A reliable fluorescent immunoassay with magnetic nanoparticle (Method A) for the quantitative determination of progesterone in cow milk was developed. This immunoassay was based on the immobilization of monoclonal anti-progesterone-antibody (mAb) on the amino modified magnetic nanoparticles (MNPs-NH_2). Both progesterone in the sample and Progesterone-3-(O-carboxymethyl) oxime-bovine serum albumin-fluorescein isothiocyanate (Prog-3-CMO-BSA-FITC) conjugate competed for the immobilized antibody. The proposed Method A was compared with other three methods (B, C, D) with different configuration of immunoreactions. The Method B is similar to Method A, but sample and conjugate were added sequentially. The Method C was based on the immobilization of Prog-3-CMO-BSA conjugate on the MNPs-NH_2, which competed with the progesterone in the sample for binding to the added mAb. The binding of mAb to the Prog-3-CMO-BSA-MNPs-NH_2 was detected using a target secondary IgG-FITC antibody. The Method D was based on immobilization of secondary antibody on the MNPs-NH_2. The mAb was immunoadsorbed to the immobilized secondary antibody. The progesterone in the sample and Prog-3-CMO-BSA-FITC conjugate competed for binding to mAb. It was found that Method A provided better analytical characteristics. Real milk samples (UHT and raw milk) were investigated for the presence of progesterone by using Method A.

INTRODUCTION

Progesterone is a steroid hormone produced mainly by corpus luteum. Progesterone plays an important role during menstrual cycles in controlling associated organs, preparing the endometrium for implantation of the fertilized

ovum, supporting pregnancy and preparing mammary glands for milk secretion (Pope and Swinburne, 1980). The progesterone concentration in blood correlates closely with the corresponding concentration of the hormone in the milk. In absolute values, progesterone concentration in milk is higher than that in serum due to its solubility in milk fat. The determination of progesterone concentration in milk samples instead of that in serum samples can be used in evaluating the reproductive condition of dairy animals. The determination of progesterone in milk has the advantage of simplicity of the sample collection and the direct use of milk sample for estimation unlike blood samples, which require the isolation of the serum before estimation. Several immunochemical methods have been developed based on isotope, enzyme and fluorescent markers for the determination of progesterone levels in serum and milk (Allen and Redshaw, 1978; Sauer et al., 1986; Kakabacos and Khosravi, 1992; Yoon et al., 1993; Claycomp et al., 1998). The use of magnetic nanoparticles with high surface area as a carry for immobilized antibody greatly improves the performance and the rate of the immunological reaction and decreases the antibody immobilization time. The Immunomagnetic bead separation technology employs immunoactivity-linked magnetic particle as a solid suspension via a specific interaction of antibody with its antigen forming a stable bond to separate the targeted molecules (Tsai et al., 2006; Zhao and Lin, 2005). More importantly, the external magnetic field could help to separate the targeted molecule from a complex environment. The cited advantages of magnetic nanoparticles lead to the development of an integrated method to provide a better sensitivity, short immobilization time, rapid analysis and simple washing procedures.

The main objective of our work was to develop a simple, rapid and reliable magnetic based-fluorescent immunoassay for determination of progesterone in milk. The method was compared with other three methods with different conjugates and different configuration of immunoreaction. The proposed method was used for detection of progesterone in milk samples.

MATERIALS AND METHODS

Reagents

All experiments were carried out using analytical grade reagents. Deionised ultrapure water (resistivity 18.2 MΩ/cm) was used throughout the work.

Progesterone-3-(O-carboxymethyl) oxime, Progesterone, N-Hydroxy-succiniimide, bovine serum albumin, albumin, from chicken egg white, dimethylformamide, Tween 20, monoclonal anti-progesterone antibody produced in rat P1922, Fluorescein 5 (6) -isothiocyanate, Isomer I, 98% (FITC), anti-rat IgG (whole molecule) antibody in rabbit, anti-rat IgG (whole molecule) -FITC antibody in rabbit were purchased from Sigma-Aldrich (St. Louis, MO, USA). N-(3-Dimethylaminopropyl)-N'-Ethyl-carbodiimide hydrochloride, glutardialdehyde 25% were supplied from Merck. Sephadex G-25 was

purchased from Pharmacia Fine Chemicals AB Uppsala, Sweden. Buffers were prepared according to standard laboratory procedure.

Preparation of progesterone-3-(O-carboxymethyl) oxime-bovine serum albumin conjugate

Progesterone-3-O-carboxymethyloxime (Prog-3-CMO) was coupled to BSA by an active ester method with some modification. Dioxin and dimethylformamide (400 µl of each) were added to 5 mg of Prog-3-CMO. To this solution,100 µl of deionized water containing 2.8 mg N-Hydroxysucciniimide and 5.2 mg N-(3-Dimethylaminopropyl)-N'-Ethyl-carbodiimide hydrochloride was added. The reaction mixture was vortex-mixed for 3h at room temperature and incubated overnight at 4ºC. 42 mg BSA was then dissolved in 2 ml of deionized water. The activated steroid was added slowly to an ice-cold solution of BSA with shaking and further incubated overnight at 4ºC. After incubation the reaction mixture was passed through a Sephadex G-25 column (1.5 x 33cm). The column was equilibrated with phosphate buffer (10 mM pH 7.4). The fractions of 1.0 ml were collected. The optical density of the obtained conjugate was measured at a wavelength of 249 nm using a Jenway 6900 UV/Vis spectrophotometer. The conjugate was freeze-dried and stored at -18ºC.

Preparation of Fluorescence-Labelled Prog-3-CMO-BSA conjugate

Fluorescein 5(6)-isothiocyanate (FITC), as a fluorescent label, was used. 15 mg of the PG-3-CMO-BSA conjugate was dissolved in 2ml deionised water. To this solution, 500µl of DMF containing 1.5 mg FITC was added slowly and then reaction mixture was incubated overnight at 4ºC. The labeling was carried out in brown vial to prevent photo-degradation of the fluorescent compound. The obtained conjugate (Prog-3-CMO-FITC) was purified by gel filtration through Sephadex 25 column. The derivatized compound was freeze-dried for 6 hours and stored at -18ºC.

Direct immobilization of anti-progesterone antibody onto the magnetic nanoparticles for Method A and Method B

The preparation and functionalization of magnetic nanoparticles were carrying out by methods described in the paper (Gabrovska et al. 2013). One ml functionalized magnetic nanoparticles with concentration 5 mg/ml^{-1} were collected by a magnet and then were added to 1 ml glutaraldehyde solution (5% w/v in 50 mm phosphate buffer solution, pH 8.0). The mixture was left for incubation 2h at room temperature with orbital agitation. The stable Schiff base was formed between an amino group of modified magnetic nanoparticles and aldehyde group of glutaraldehyde in basic medium, pH 8.0. The particles were washed five times with phosphate buffer solution (pH 7.4). Then 10 µl of 15 mg/ml^{-1} antibody in 1 ml of 10 mm phosphate buffer solution pH 7.4 was added to the magnetic nanoparticles and the covalent immobilization was carried for 2.5h at 4ºC. The derivatized antibody-magnetic particles were collected with a magnet and washed three times with the phosphate buffer solution (pH 7.4) and resuspended in 1 ml of

the same buffer. Then magnetic nanoparticles-antibody were incubated with 1 ml of 0.25M sodium cyanoborohydride in 10 mm phosphate buffer solution, pH 7.4. After 30 min at 37ºC, the magnetic nanoparticles-antibody was washed three times with 10 mm phosphate buffer solution, pH 7.4. 10 mm phosphate buffer solution (pH 7.4) with 5 % bovine serum albumin and 0.05% Tween 20 was added to the antibody-magnetic nanoparticles to block the unreacted active groups (Puertas et al., 2010) and the mixture was gently stirred for 1 hour at room temperature. The magnetic nanoparticles–Ab were washed four times with 10 mm phosphate buffer solution (pH 7.4) and resuspended in 1 ml of the same buffer to reach 5 mg/ml^{-1} stock solution.

Direct immobilization of Prog-3-CMO-BSA conjugates onto the magnetic nanoparticles for Method C

One ml functionalized magnetic nanoparticles with concentration 5 mg/ml^{-1} were collected by a magnet and then were added to 1 ml solution of glutaraldehyde (5% w/v in 50 mm phosphate buffer solution, pH 8.0). The mixture was left for incubation 2 h at room temperature with orbital agitation. The stable Schiff base was formed between an amino group of modified magnetic nanoparticles and aldehyde group of glutaraldehyde in basic medium, pH 8.0. The particles were washed five times with phosphate buffer solution (pH 7.4). Then 1 ml of 0.5 mg/ml^{-1} Prog-3-CMO-BSA conjugate in 10 mm phosphate buffer solution pH 7.4 was added to the magnetic nanoparticles and the covalent immobilization was carried for 2.5h at 4ºC. The immobilized magnetic particles were collected with a magnet and washed three times with the phosphate buffer solution (pH 7.4) and resuspended in 1 ml of the same buffer. Then magnetic nanoparticles-Prog-3-CMO-BSA was incubated with 1 ml of 0.25M sodium cyanoborohydride in 10 mm phosphate buffer solution pH 7.4. After 30 min at 37ºC, the magnetic nanoparticles-antibody was washed three times with 10 mm phosphate buffer solution, pH 7.4. 10 mm phosphate buffer solution (pH 7.4) with 5 % OVA and 0.05% Tween 20 was added to Prog-3-CMO-BSA-magnetic nanoparticles to block the unreacted active groups (Puertas et al. 2010) and the mixture was gently stirred for 1 hour at room temperature. The magnetic particles–Prog-3-CMO-BSA were washed four times with 10 mm phosphate buffer solution buffer (pH 7.4) and resuspended in 1 ml of the same buffer to reach 5 mg/ml^{-1} stock solution.

Direct immobilization of anti-rat IgG antibody onto the magnetic nanoparticles for Method D

One ml functionalized magnetic nanoparticles with concentration 5 mg/ml^{-1} were collected by a magnet and then were added to 1 ml solution of glutaraldehyde (5% w/v in 50mm phosphate buffer solution, pH 8.0). The mixture was left for incubation 2 h at room temperature with orbital agitation. The stable Schiff base was formed between the amino group of modified magnetic nanoparticles and an aldehyde group of glutaraldehyde in basic medium, pH 8.0. The particles were washed five times with phosphate buffer solution (pH 7.4). Then 10 µl

of 4 mg/ml^{-1} anti-rat IgG antibody in 10 mm phosphate buffer solution, pH 7.4 was added to the magnetic nanoparticles and the covalent immobilization was carried for 2.5h at 4ºC. The derivatized secondary antibody-magnetic particles were collected with a magnet and washed three times with the phosphate buffer solution (pH7.4) and resuspended in 1 ml of the same buffer. 10 µl of 15 mg/ml^{-1} monoclonal anti-progesterone antibody in 1 ml of 10 mm PBS, pH 7.4 was added to magnetic nanoparticles-anti-rat IgG antibody and incubated for 2.5h at room temperature. The magnetic particles were collected with a magnet and washed three times with the phosphate buffer solution (pH 7.4). 10 mm PBS (pH 7.4) with 5 % bovine serum albumin and 0.05% Tween 20 was added to magnetic nanoparticles-anti-rat IgG antibody-anti-progesterone antibody to block the unreacted active groups (Puertas et al. 2010) and the mixture was gently stirred for 1 hour at room temperature. The obtained complex magnetic particles-anti-rat antibody-anti-progesterone antibody was washed four times with 10 mm phosphate buffer solution buffer (pH 7.4) and resuspended in 1 ml of the same buffer to reach 5 mg/ml^{-1} stock solution.

Immunoassay analysis by Method A

The principle of the immunoassay procedure utilizing the one-step incubation assay is presented in Figure 1.

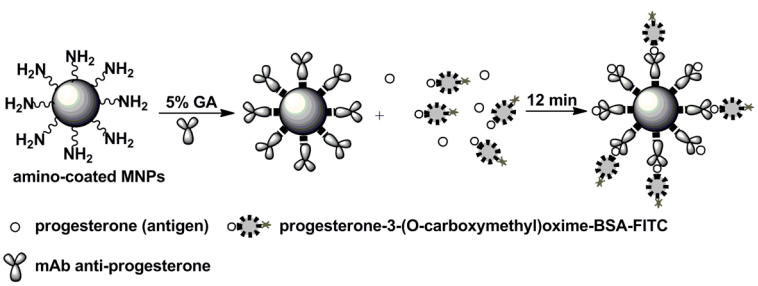

Figure 1. Principle of immunoassay analysis, Method A.

75 µl of 5 mg/ml^{-1} MNPs-mAb were transferred to tubes and collected by a magnet. The supernatant was pipetted out. 60 µl of progesterone working standards (0-100 ng/ml^{-1}) and 100 µl Prog-3-CMO-BSA-FITC (7.5 µg/ml^{-1}) conjugate was added to MNPs-Ab and incubated for 12 min at 37ºC. Both progesterone in the sample and Prog-3-CMO-BSA-FITC conjugate competed for the immobilized antibody. The unbound fluorescent labelled conjugate (supernatant) was separated. The supernatant was diluted to 450 µl and the fluorescence was measured by Cary Eclipse fluorescence spectrophotometer (Varian, AU, USA). The used fluorescent labelled conjugate shows highest emission in the green region of the visible-light spectrum. Experimental signals were normalized using the following expression:

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

where **B** is the signal (intensity of fluorescence) measured in the presence of the increasing analyte concentrations; **Bx** is the signal in the absence of progesterone; and $\mathbf{B}_0$ is the signal of the initial conjugate solution.

Immunoassay analysis by Method B

The principle of the immunoassay procedure utilizing the two-step incubation assay is presented in Figure 2.

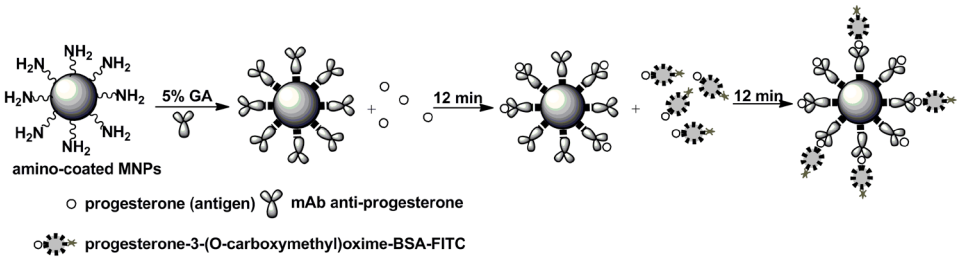

Figure 2. Principle of immunoassay analysis, Method B.

75 µl of 5 mg/ml^{-1} MNPs-Ab were transferred to microfuge tubes and collected by a magnet. The supernatant was pipetted. 60 µl of each sample with different progesterone concentrations (0 to 100 ng/ml^{-1} in progesterone-free milk) were added to MNPs-Ab and preincubated for 12 min at 37ºC to allow the progesterone to bind with antibody. Fluorescent labelled conjugate (100 µl) was added and further mixed and incubated for 12 min at 37ºC. The unbound fluorescent labelled conjugate (supernatant) was separated. The supernatant was diluted to 450 µl and the fluorescence was measured by the same procedure as described in Method A.

Immunoassay analysis by Method C

75 µl of 5 mg/ml^{-1} MNPs-Prog-3-CMO-BSA were transferred to microfuge tubes and collected by a magnet. The supernatant was pipetted. 60 µl of each sample with different progesterone concentrations (0 to 100 ng/ml^{-1} in progesterone-free milk) and 60 µl of anti-progesterone antibody (2.75 µg/ml^{-1}) were added to MNPs-Prog-3-CMO-BSA and incubated for 30 min at 37ºC.

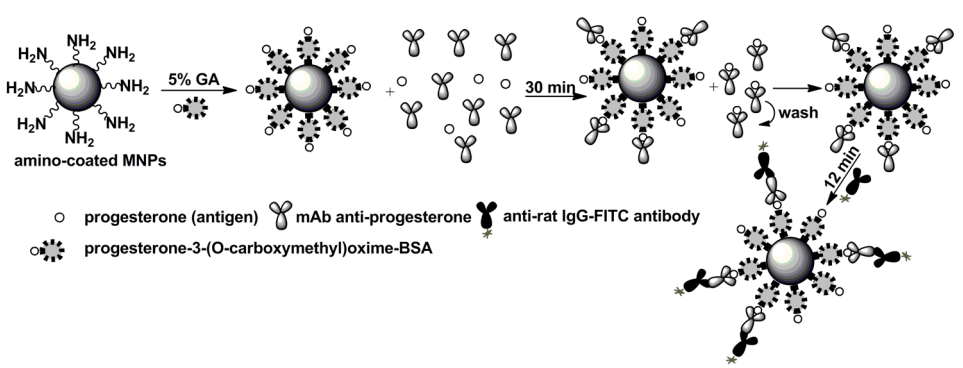

Figure 3. Principle of immunoassay analysis, Method C.

The magnetic nanoparticles were washed three times with 10 mm PBS, pH 7.4 and added 100 µl an anti-rat IgG-FITC antibody and incubated for 12 min at 37ºC. The unbound fluorescent labelled antibody (anti-rat IgG-FITC) was separated, and the fluorescence was measured the same procedure as described in Method A.

Immunoassay analysis by Method D

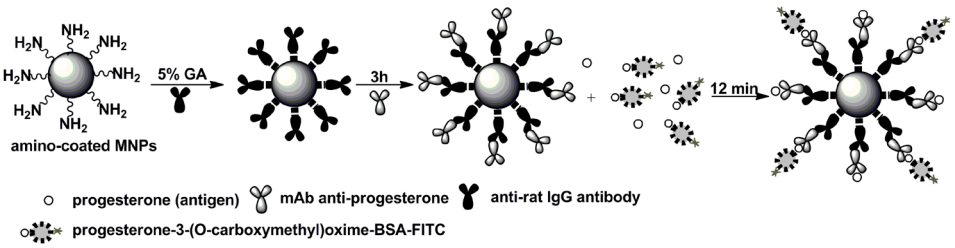

Figure 4. Principle of immunoassay analysis, Method D.

Method D was prepared the same procedure as described in Method A, but immobilization onto magnetic nanoparticles is different.

Preparation of progesterone standards in cow's milk (raw and UHT)

Full-fat cow's milk in estrus (progesterone-free measured by commercial progesterone ELISA kit-EuroProxima, Netherlands) were used for the preparation of milk standards. Progesterone standards were prepared by adding a known amount of progesterone (0.05, 0.25, 1.00, 2.00, 5.00, 10.0, 25.0, 50.0, 100 ng/ml^{-1}) to the progesterone-free milk. The applicability of the developed four immunoassays to real sample analysis was investigated by analysing of two types of milk: raw and UHT cow's milk. It should be noted that it was not necessary to perform any sample pretreatment to the milk samples.

RESULTS AND DISCUSSION

The obtained four immunoassays for the determination of progesterone were investigated by analysing of two types of milk: raw and UHT cow's milk. The magnetic-based fluorescent immunoassay by Method A was compared with other three methods (B, C, D). All methods were based on the use of magnetic nanoparticles as a solid support for covalently immunoreagents immobilization. The nanoparticles' size allows the possibility of handling the particles in suspension. A comfortable separation of bound and free fraction of the tracer can be performed only through a simple collection of the magnetic particles by a permanent magnet (Tudorache et al., 2005). Other advantages of the magnetic beads compared to the conventional solid supports used in immunoassays are easy manipulation, low pressure drop, high-mass transfer rate, good fluid-solid contact, perspectives for system automation and miniaturization (Zhao and Shippy 2004). The choice of

solid phase and the separation procedure is important to enhance the sensitivity in the heterogeneous enzyme immunoassays. Thus, the development of a new magnetic particle-based enzyme-linked immunosorbent assay for determination of progesterone in milk is very interesting. Application of MNPs helps to eliminate non-specific binding, retain higher activity of immobilized biomolecules, and stabilize the binding between the solid phase and proteins.

The immunoassays for the determination of progesterone in raw cow's milk were performed by four investigated methods. The experiments were carried out with progesterone standard solutions in raw milk (progesterone-free milk). The results for determination of the progesterone in raw milk by Method A, B, C and D are presented in Figure 5. The based characteristics of four different immunoassays were compared.

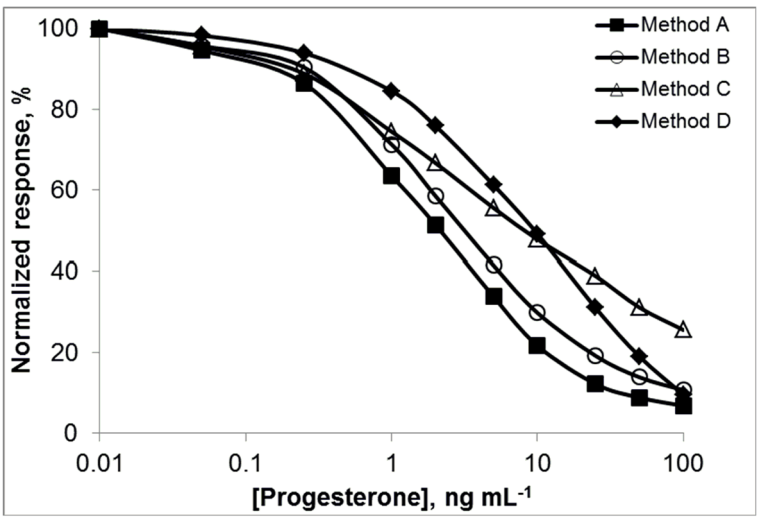

Figure 5. Calibration curves of progesterone in raw milk, using fluorescent immunoassay with four different configurations of immunoreactions.

Calibration curves were prepared by plotting progesterone concentrations against the fluorescence signals. An ideal matrix for the calibration would be milk of an animal in estrus, as there is no progesterone presented in the milk. Figure 5 shows typical calibration curves obtained for progesterone standards in raw milk at concentrations ranging from 0.05 to 100 ng/ml^{-1} by using Method A, B, C, and D. The analytical parameters for the detection of progesterone in raw milk obtained by four methods are presented in Table 1. The linearity of the standard curve using Method A was from 0.25 to 10 ng/ml^{-1}. The assay sensitivity is usually expressed in terms of its lower detection limit (LOD) and slope of the curve. The LOD is the lowest concentration of analyte giving a response statistically different from that observed in the absence of the analyte. It is

calculated as 95% from B_0 (Simersky et al., 2007). The detection sensitivity of progesterone in raw milk by method A was low - 0.045 ng/ml^{-1}.

The linear range of the curve using Method B is the same, but the limit of detection was higher 0.065 ng/ml^{-1}. For the methods C and D, the linear range of the curves was wider, respectively, 0.25-100 ng/ml^{-1} and 1-100 ng/ml^{-1}. The detection sensitivity of progesterone in raw milk by method D was the highest – 0.2 ng/ml^{-1}. A good correlation was obtained with satisfied correlation coefficients R2 for all immunoassays (Table 1). The influence of the applied different methods on the sensitivity of the analysis was studied. Results in Table 1 showed that Method A was the lowest detection limit and the highest sensitivity. Probably, the degree of immobilization of mAb on MNPs at method A was higher than the immobilization degree of Prog-3-CMO-BSA conjugate on MNPs at method C and secondary anti-rat IgG antibody on MNPs at method D. Beside that, the competitive immunoassay method A was given better results than consequently, method B.

Table 1. The linear equations and correlation coefficients of calibration curves for progesterone in milk, using fluorescent immunoassay with four different configurations of immunoreaction.

Method	***Linear range, ng/ml^{-1}***	***Linear equation***	***R^2***	***LOD, ng/ml^{-1}***	***Total assay time, min (min)***
Method A, raw milk	0.25 – 10	y = -17.69ln(x)+62.739	0.9991	0.045	12
Method B, raw milk	0.25 – 10	y = -16.89ln(x)+86.597	0.9969	0.065	24
Method C, raw milk	0.25-100	y = -10.78ln(x)+73.755	0.9985	0.06	42
Method D, raw milk	1-100	y = -16.67ln(x)+69.032	0.9955	0.2	12
Method A, UHT milk	0.25 – 10	y = -17.79ln(x)+53.76	0.9993	0.03	12

The selected Method A was applied to evaluate progesterone in two types of milk (raw and UHT). A series of standard solutions with different progesterone concentrations were prepared in UHT milk and raw milk. Milk samples were directly analysed with the selected immunoassay, without additional sample pre-treatment or analyte extraction. The linear range of the standard curve in UHT milk was the same like in raw milk, from 0.25 to 10 ng/ml^{-1} progesterone (Figure 6). A good correlation was obtained with a satisfied R2 0.9993 and 0.9991 for UHT and raw milk, respectively (Table 1). The LOD of analysis in UHT milk was less than LOD of progesterone in raw milk. Obviously, the fat in UHT milk was

less than the fat in raw milk, and this was the reason for the highest sensitivity of progesterone immunoassay in UHT milk.

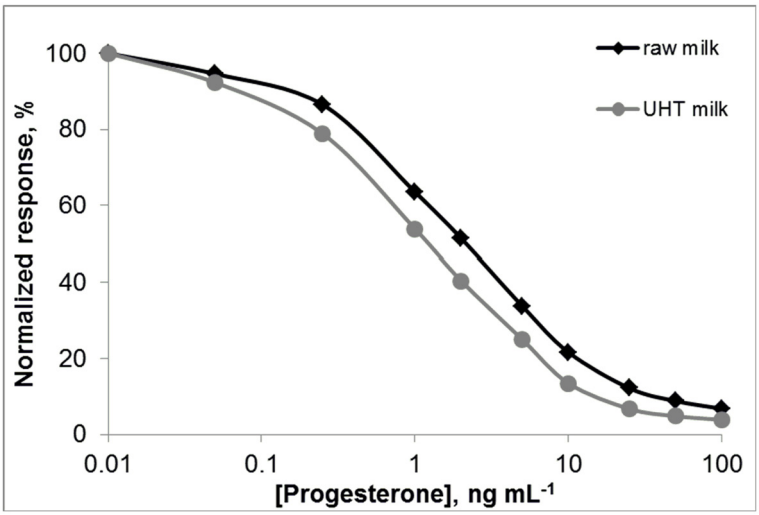

Figure 6. Calibration curves of progesterone in raw and UHT milk, using fluorescent immunoassay with the configuration of immunoreaction by method A.

The four immunoassays for detection of progesterone in milk with different configuration of immunoreactions were compared. It was found that the competitive fluorescent immunoassay based on magnetic nanoparticle with configuration – Method A showed excellent sensitivity (LOD<0.045 ng/ml^{-1}). Probably, the degree of immobilization of mAb on MNPs at method A was higher than the immobilization degree of PG-3-CMO-BSA conjugate on MNPs at method C and secondary anti-rat IgG antibody on MNPs at method D. Besides that, the competitive immunoassay by method A was given better results than consequently, immunoassay by method B.

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