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Original Research Article

Rapid time-resolved fluoroimmunoassay for diethylstilbestrol in cow milk samples with a highly luminescent Tb^{3+} chelate

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ABSTRACT

Diethylstilbestrol (DES) is a non-steroidal synthetic molecule with estrogenic activity that provokes increased health risk in women whose mothers were given DES during pregnancy. In spite of this, it is sometimes illegally used as growth promoter in livestock breeding. Therefore methods that make it possible to monitor the presence of DES in food or in the environment might be of great interest. We describe a quantitative immunoassay with highly luminescent Tb^{3+} complex of 10-[4-(3-isothiocyanatopropoxy)benzoylmethyl]-1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid ($Tb^{3+} \subset \mathbf{1}$) conjugated with a goat anti-rabbit IgG applied as marker in a time-resolved determination of DES. Lanthanide chelate is very stable and highly luminescent in aqueous solution, and makes it possible to reach the 0.5 $\mu g\,L^{-1}$ sensitivity required in the control of illegal use of DES. DES recovery in milk samples was between 96% and 104.2%. The assay was performed using a rabbit polyclonal antibody specific to DES. This procedure can be applied directly to raw milk samples, and is useful as an alternative to conventional methods, providing a simple, on-site control for dairy farms.

1. Introduction

Diethylstilbestrol (DES) is a non-steroidal synthetic molecule with estrogenic activity, which has been used fore more than forty years to prevent human pregnancy complications, such as miscarriage, pre-term delivery and intrauterine fetal demise. Research has shown that DES is responsible for a higher incidence of vaginal and cervical adenocarcinoma as well as an increased risk of infertility in women whose mothers had taken DES during pregnancy (Kaufman et al., 1980; Giusti et al., 1995; Goldberg and Falcone, 1999; Palmer et al., 2001; Schrager and Potter, 2004). However, because of its anabolic effects, it is also used as growth promoter in animal breeding. Many natural or synthetic estrogens possess anabolic properties because they accelerate animal growth by increasing muscle mass (Paris et al., 2006).

The use of stilbenes and other anabolic hormones in animal breeding is banned by European Community legislation (European Commission, 1996). These compounds present a potential health risk because they can be transferred not only to milk and other food products from treated animals, but also to the environment, causing adverse consequences associated with a consumption of food products (Daxenberger et al., 2001; Hong et al., 2004) or exposure to residues contaminating the environment (Tapiero et al., 2002; Lopez de Alda et al., 2002; Hájková et al., 2007; Newbold et al., 2009).

Among synthetic estrogens, DES is one of the most active molecules (Leffers et al., 2001) and as it is illegal in many countries, its presence in the milk or in food products must be carefully controlled. To this end several analytical approaches such as gas-chromatography (Hájková et al., 2007; Dickson et al., 2009), liquid chromatography–mass spectrometry (Lopez de Alda et al., 2002; Msagati and Nindi, 2006; Schmidt et al., 2008; Wang et al., 2008; Kaklamanos et al., 2009; Yang et al., 2009) bioassays (Bovee et al., 2006) and immunological assays (Elliot et al., 1994; Arts et al., 1998; Ohno et al., 2002; Koda et al., 2002; Dickson et al., 2003; Xu et al., 2006; Zhao et al., 2009) have been developed for the DES detection in biological fluids, tissues and a variety of other matrices.

Analyses of DES by conventional methods are based on separative techniques that isolate the target compound from the

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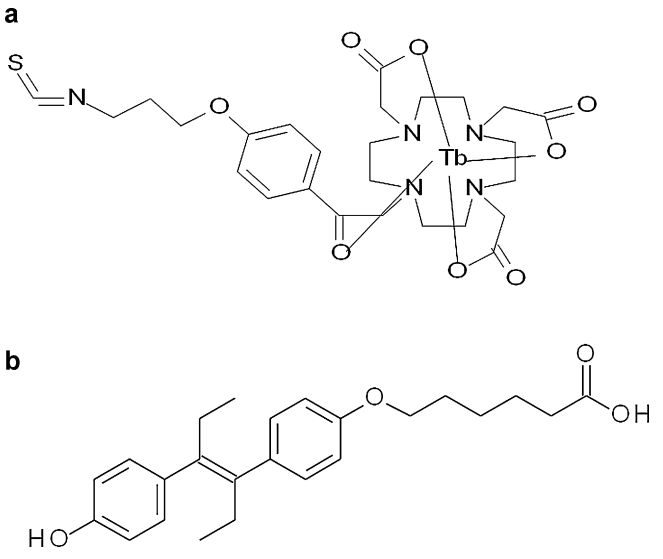

Fig. 1. (a) Structural formula of 10-[4-(3-isothiocyanatopropoxy)benzoylmethyl]-1,4,7,10 tetraazacyclododecane-1,4,7-triacetic acid Tb(III) complex (Tb3 + Ì1). (b) Structural formula of 6-{4-[1-ethyl-2-(4-hydroxy-phenyl)-but-1-enyl]-phenoxy}-hexanoic acid (DESHA).

matrix. In these cases, an internal standard is necessary to monitor the recovery and interfering peaks, and baseline noise in the chromatograms must be reduced to increase sensitivity. Furthermore, the screening of numerous samples by conventional methods is very expensive because they are processed one at a time, often using laborious and time-consuming procedures. Moreover, each step of the analytical process can introduce errors into the final result. In this framework, immunological methods would appear to present a more convenient and advantageous approach for DES determinations, because they cover a wide concentration range and do not require sample pretreatment. These methods are simple, rapid, sensitive and specific. Furthermore, many samples can be analyzed simultaneously in a short incubation time.

In this paper we report the application of goat anti-rabbit IgG conjugate with high luminescent lanthanide chelate, Tb^{3+} complex of 10-[4-(3-isothiocyanatopropoxy)benzoylmethyl]-1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid ($Tb^{3+} \subset$ **1**) (Fig. 1a), as marker (Bacigalupo et al., 2009) in a time-resolved fluoroimmunoassay (TR-FIA) for quantitative determination of DES. The high fluorescence signal of the lanthanide chelate and the time-resolved reading mode eliminate the fluorescence background, and it can moreover be applied directly to raw milk samples.

2. Materials and methods

2.1. Materials

Standard DES (≥99%, HPLC) and the chemical related cross-reacting compounds (17-α-ethynylestradiol, testosterone, hexestrol, dienestrol, 17-α-estradiol, estriol, zeranol, zearalenone), and all other chemicals, including bovine serum albumin (BSA), chicken egg albumin (OVA), carrageenan, and goat anti-rabbit IgG, were obtained from Sigma–Aldrich (Milan, Italy). Specific polyclonal antibody raised in rabbit was purchased from AbD Serotec (Oxford, UK). Luminescent $Tb^{3+} \subset$ **1** complex was synthesized through a multistep procedure as reported in Scalera (2008). The product was characterized by electrospray ionization–mass spectrometry (ESI–MS) m/z 758.12719 [M + Na]+, calculated for $C_{26}H_{34}N_5O_8STbNa$ m/z 758.12737 and Infrared Spectroscopy IR (KBr) ν = 2190.74 cm^{-1} (–NCS).

2.2. Apparatus

A single-photon-counting time-resolved fluorometer (1232 DELFIA Fluorometer; Wallac, Turku, Finland) was used to measure fluorescence.

2.3. Specimen collection

The milk samples were collected in glass vials from lactating cows, and stored at −20 °C until use. Milk samples with a different protein and lipid profile were obtained from cows raised under different environments and feeding conditions. Pooled milk was obtained by mixing 100 mL of milk derived from milking 10 cows. Using a pool of milk rather than milk from a single cow avoided any possible interference that could have arisen from the lipid and protein composition in the milk from one single animal. The pool was fractionated in glass vials, stored at −20 °C, and treated in each experiment in the same way as other samples. The deep-freeze process does not affect the assay performance (Bacigalupo et al., 2008).

2.4. Syntheses

Hapten. 6-{4-[1-Ethyl-2-(4-hydroxy-phenyl)-but-1-enyl]-phenoxy}-hexanoic acid (DESHA) was obtained from ethyl 4-bromobutyrate intermediate as reported by Johnson et al. (1979) with some modifications as described below. The structural formula is reported in Fig. 1b.

A sample of ethyl 4-bromobutyrate (1.3 mL in 60 mL of anhydrous tetrahydrofuran) was slowly added to a suspension of DES (7.4 mmol) dissolved in 60 mL of anhydrous methanol: tetrahydrofuran (4:1) containing 3.1 g of solid potassium carbonate maintained at 60 °C under magnetic stirring. The reaction mixture was kept in the dark at room temperature for 72 h, then filtered and the solvent evaporated *in vacuo*. Purification of the crude solvent by column chromatography (2 cm × 30 cm) on silica gel (230–400 mesh) eluted with 10% acetone in petroleum ether produced 1.1 g of pure 6-{4-[1-ethyl-2-(4-hydroxy-phenyl)-but-1-enyl]-phenoxy}-hexanoic acid ethyl ester. This compound was characterized by the melting point (m.p.) 132–134 °C and showed as a single spot, Rf = 0.81 on thin layer chromatography (TLC) plates (silica gel F254) (petroleum ether/acetone 4:1). A solution of this compound in 50 mL of methanol and 10 mL of 2 M NaOH was heated at reflux for 1 h, brought to pH 5 with 1 M sulfuric acid, diluted to 200 mL with water and than extracted with ethyl acetate. The solvent was removed to yield 980 mg of the product that was unequivocally characterized by means of TLC; Rf = 0.3 (petroleum ether/acetone 4:1); m.p. 160–163 °C.

DESHA-OVA. DESHA-OVA was synthesized and used as solid phase on polystyrene micro-wells. Briefly 6.8 mg of DESHA were dissolved in 0.3 mL of anhydrous dimethylformamide containing 7 μL of tri-n-butylamine and cooled to 4 °C. After 5 min 18 μmol of isobutylchloroformate were added and the solution was left for 30 min at 4 °C. This mixture was added to 3 mg of OVA dissolved in 0.5 mL 40% dimethylformamide in water and then 50 μL 1 N NaOH was added. Reaction mixture was stirred at 4 °C for 1 h while the pH was kept at 8.5–9.0 by repeatedly adding 5 μL 1 N NaOH, and then left at 4 °C overnight. The conjugate was purified by gel filtration on Sephadex G-50 column with 0.05 M NH_4HCO_3 and lyophilized.

IgG-($Tb^{3+} \subset$ **1**). $Tb^{3+} \subset$ **1** was conjugated with a goat anti-rabbit IgG. The conjugate was prepared using 2 mg of affinity purified IgG dissolved in 2 mL of 0.1 M sodium carbonate buffer (pH 9). A solution of 500 μg of chelate in 100 μL of ethanol was then added and the mixture was incubated for 30 min at room temperature. The labeled IgG were separated on Sephadex G-50 column, eluting