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IMMUNOAFFINITY CHROMATOGRAPHY AND A BIOTIN-STREPTAVIDIN AMPLIFIED ENZYMEIMMUNOASSAY FOR SENSITIVE AND SPECIFIC ESTIMATION OF ESTRADIOL-17β

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Summary—A sensitive test system has been developed for estimation of estradiol-17β (E_2) in bovine plasma. Plasma extracts are first purified by a selective immunoaffinity chromatography (IAC) using an antibody raised against estradiol-6-carboxymethyloxime-bovine serum albumin and immobilized to Sepharose. The eluate was analysed by a competitive enzyme immunoassay (EIA) on microtitration plates. For the assay the wells of microtitration plates were coated with affinity purified sheep IgG (antirabbit IgG) that binds the hormone specific antibody raised in rabbits against estradiol-17-hemisuccinate-bovine serum albumin. E_2 is estimated by displacement of biocytinyl-E_2, that was produced by ligation of estradiol-17β, D-glucuronic acid and biocytin. Bound biocytinyl-E_2 is detected after binding of streptavidin-peroxidase and colour production by the enzyme. A very high amplification was possible with this technique and the absolute detection limit amounted to $\approx$120 fg/well at 94% relative binding. By combination of IAC and EIA the following levels of E_2 were found in bovine plasma: male or female calves $<$2.7 pg/ml, cycling cow 0.5–7 pg/ml, cow during last month of pregnancy 9–310 pg/ml, mature bull 5–30 pg/ml. However, up to 1110 pg E_2/ml were found in plasma of a calf after treatment with an illicit hormone preparation used for growth promotion; after 21 days levels declined to 6 pg/ml which is hardly different from controls. In conclusion, the IAC/EIA can be used for sentitive estimation of estradiol-17β in plasma from all type of cattle and for control of improper use of E_2 after commitment of a threshhold level.

INTRODUCTION

Levels of estradiol-17β (E_2) in peripheral bovine plasma are very low. In calves not more than 1–2 pg/ml are present [1] and in cycling females there is a variation between 1–5 pg/ml [2]. Only in plasma from pregnant animals [3] or from animals after illegal treatment with anabolic agents [4] levels between 100 and 1000 pg/ml may be found. Mainly radioimmunoassays (RIA) have been used for the estimation of those very low levels, but it is hardly possible to obtain absolute sensitivities better than 1–2 pg, when [^{3}H]E_2 is used as a label. Therefore a few milliliters of plasma were needed to be extracted and processed with complicated methods prior to the assay. Enzymeimmunoassays with better sensitivity than RIA [5–7] have not been validated for the estimation of E_2 in plasma of domestic animals.

It was our aim to develop a test system that involves an efficient, quick prepurification system and a good assay sensitivity in the sub-picogram range with minimum interferences of other estrogens or any other substance. For this purpose an immunoaffinity chromatography (IAC) and a biotin-streptavidin amplified EIA were developed. For the two steps antibodies with different selectivities seemed to be useful and for EIA a conjugate was synthesized that was able to form a bridge between E_2-antibody and streptavidin peroxidase in order to get high amplification.

EXPERIMENTAL

Purification of anti-E_2 immunoglobulins and linkage to Sepharose

A rabbit antiserum (Code E2/2; binding capacity $\approx$ 1 μg E_2/ml) was raised against 17β-estradiol-6-carboxymethyloxime-bovine serum albumin (E_2-6-CMO-BSA), the immunoglobulins were precipitated by addition of 180 mg sodium sulfate/ml serum and sedimented by centrifugation (3000 *g*, 20°C, 20 min). The sediment was washed twice with 5 ml 18% sodium sulfate, dissolved with 5 ml 0.1 M sodium carbonate pH 8.5, dialysed overnight against the latter buffer, the undissolved material was removed by centrifugation and the dissolved immunoglobulins used for coupling to Sepharose.

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15 g wet Sepharose CL-4B (Pharmacia, Freiburg, F.R.G.) were washed in a glass suction filter with 100 ml deionized water plus 100 ml 67 mM phosphate buffer pH 11.0, transferred into a beaker and filled up to 30 ml with phosphate buffer. The mixture was stirred while 4.5 g powdered cyanogen bromide were added. Temperature between 18 and 22°C and pH 10.5–11.0 were observed by adding crushed ice and 8 M NaOH. After 10 min proton release ended and the gel was washed in a suction filter with 200 ml chilled 0.1 M $NaHCO_3$ pH 8.5. The activated gel was combined with the purified immunoglobulins in a beaker, filled up to 30 ml with carbonate buffer, stirred for 2 h at 0°C and left overnight at 0°C without stirring. The material was washed with 600 ml carbonate buffer and with 100 ml 20 mM Tris–acetate pH 7.5/methanol 20/80 v/v for removing bound steroids from the antibody (last step no suction). Finally the gel was equilibrated with 20 mM Tris–acetate pH 8.5/methanol 80/20 v/v and stored in this buffer.

Preparation of affinity purified sheep IgG

A small column containing 5 g rabbit IgG agarose gel (Sigma) was prepared and 15–20 ml clear plasma containing 6 mM EDTA, from a sheep immunized with rabbit IgG were applied to the column. The gel was washed with 10 ml 0.5 M NaSCN pH 8.0 followed by 10 ml 0.1 M glycine–HCl pH 3.5 and eluted with 15 ml 0.1 M glycine–HCl pH 2.0 into a vial containing 2 ml 1 M Tris–HCl pH 8.0 (all steps at room temperature). The eluate was immediately dialysed against 66 mM NaH_2PO_4/Na_2HPO_4 pH 7.2 and the IgG obtained quantified by the biuret procedure.

Preparation of biocytinyl-estradiol

Synthesis. 20 μmol 17β-Estradiol-17β,D-glucuronic acid plus 20 μmol *N*-hydroxysuccinimide were dissolved in 250 μl *N,N'*-dimethylformamide (DMF), combined with 20 μmol *N,N'*-dicyclohexylcarbodiimide dissolved in 250 μl DMF and stirred overnight at room temperature (all chemicals: Sigma, Deisenhofen, F.R.G.). In order to remove the resulting precipitate, the mixture was passed over a small filter column (product 71211; Baker Inc., Phillipsburg, N.J., U.S.A.) and the precipitate reextracted with 1 ml DMF. The total effluents were combined with 20 μmol biocytin (Sigma) dissolved in 200 μl water and stirred overnight. The mixture was diluted with 15 ml water and pumped through two combined Sep-Pak-RP18-cartridges (Waters, Königstein/Taunus, F.R.G.) with a syringe. The adsorbed product was washed with 3 ml methanol/water 1/9 v/v and eluted with 5 ml methanol/water 6/4 v/v (all steps at room temperature).

Identification. 10 μl eluate were diluted with 2 ml methanol/water 2/8 v/v and 500 μl were applied to an HPLC system (Column: LiChrospher 100, RP18, 5 μm; 50 × 4 mm precolumn and 250 × 4 mm). Elution was done with a linear gradient of methanol:20 mM Tris–acetate pH 7.2 from 40/60 v/v to 60/40 v/v within 20 min (1 ml/min; 0.3 ml/fraction). The eluted material was controlled in the u.v.-detector (284 nm) and the presence of immunoactive estradiol was measured after 1:100 dilution of the fractions by EIA as described earlier [7]. Biocytin was estimated in 100 μl of each chromatography fraction in a competitive ligand binding test using avidin from egg-white (dilution 1:300) as a specific binder. [^{14}C]Biotin (about 3000 cpm) was used as a label and biocytin as competitor (0.2–100 ng) in a test system comparable to a classical RIA. Unbound [^{14}C]biotin was removed with charcoal and the remaining bound label measured in a scintillation counter.

Purification. The eluate from the Sep-Pak-Cartridges was concentrated to 1.5 ml under reduced pressure and applied to the HPLC via a 2 ml sample loop (HPLC-system like above). Biocytinyl-estradiol was collected into a single fraction and the described procedure was repeated in order to get a complete removal of estradiol-glucuronide.

Quantification. Due to unknown crossreactivities of the product in both ligand binding tests (E_2, biocytin) a precise quantification was not possible with these tests. Therefore, biocytinyl-estradiol was measured by HPLC–u.v.-detection at 284 nm after calibration of the detector with estradiol-17β (HPLC column as described; isocratic elution: methanol/20 mM Tris–acetate pH 7.2 70/30 v/v.

Extraction and IAC of estradiol-17β

Plasma (0.5 or 1 ml) were extracted with 3 or 6 ml tert-butylmethyl ether/petrol ether 30/70 v/v, the solvent was removed at 50°C and the residue dissolved in 1 ml 20 mM Tris–acetate pH 7.2/methanol 80/20 v/v. Triplicate plasma samples spiked with [^{3}H]E_2 (about 3000 cpm) were included to monitor recovery through the whole extraction and purification process.

Wet anti-E_2-Sepharose gel (200 μg) was placed into 1 ml filter columns (Baker) and equilibrated with 1 ml 20 mM Tris–acetate pH 7.2/methanol 80/20 v/v prior to sample application. The gel was washed with 1 ml 67 mM phosphate buffer pH 7.2 and with 2.5 ml 20 mM Tris–acetate pH 7.2/methanol 60/40 v/v. After elution of E_2 with 0.8 ml 20 mM Tris–acetate pH 7.2/methanol 20/80 v/v the gel was cleaned with further 0.8 ml of the latter solution and reequilibrated with the first buffer prior to the next sample. The eluate was dried under reduced pressure, the residue reconstituted in 200 μl assay buffer (40 mM NaH_2PO_4/Na_2HPO_4, 0.15 M NaCl, pH 7.2, 0.1% bovine serum albumin) and used for the EIA.

General assay protocol for E_2 EIA

(1) Coating of affinity purified sheep IgG (anti-rabbit IgG) to wells of the microtitration plate

(No. 439 454; Nunc, DK-4000 Roskilde). 1 μg/well in 100 μl coating buffer; incubation 2 h at 22°C or overnight at 0°C; coating buffer: 50 mM $NaHCO_3$ pH 9.6.

(2) Complete coating of the wells with BSA; 0.3 mg/well in 300 μl assay buffer (40 mM NaH_2PO_4/Na_2HPO_4, 0.15 M NaCl, pH 7.2, 0.1% bovine serum albumin); incubation 15–45 min 22°C, decant; storage up to 6 months at −20°C if needed.

(3) Washing twice with 300 μl/well 0.05% TWEEN 80.

(4) Calibration curves were prepared from an estradiol standard by 1:2 dilution with assay buffer (range 15–0.12 pg/50 μl).

(5) 50 μl of standard or sample plus 200 μl antibody dilution (1:300,000) containing 10 fmol biocytinyl-E_2 were pipetted into each well by the aid of a diluter dispenser. The antibody (Code E 2/3) had been raised in rabbits against estradiol-17β-hemisuccinate-bovine serum albumin (E_2-17-HS-BSA).

(6) Incubation 18 h at 6–9°C with gentle agitation.

(7) Decanting and addition of 20 ng streptavidin-peroxidase (Sigma) in 100 μl assay buffer; incubation for 15 min at 6–9°C.

(8) Washing 4 times with 300 μl 0.05% TWEEN 80 (chilled to 0°C).

(8) Dispensing of 150 μl substrate solution and incubation for 40 min at 25°C; *substrate buffer:* 10 mM sodium acetate adjusted to pH 5.5 with citric acid; *substrate solution:* 25 ml substrate buffer plus 100 μl 1% H_2O_2 plus 400 μl 0.6% tetramethylbenzidine in dimethylsulfoxide.

(10) Addition of 50 μl stop reagent (2 M H_2SO_4).

(11) Colour reading at 450 nm with 8-channel microtitration plate photometer (Multiskan, Flow, Meckenheim, F.R.G.).

Other methods

Progesterone in plasma was measured by EIA [8]. For estimation of total steroidal estrogens in plasma conjugated steroids were hydrolysed [9], extracted (no IAC) and measured by EIA as described herein.

RESULTS

The separation of the produced material from intact E_2 and biocytin is presented in Fig. 1. Biocytin, that had been removed by the prepurification, migrates to approximately 4 min and was not detectable by E_2 assay or by u.v.-detector in the HPLC. Free E_2-glucuronide was found at 12–13 min and only at 17–18 min a substance was eluted that had both E_2 and biocytin properties. The proposed chemical structure of this new product is presented in Fig. 1. After complete purification of the material the precise quantification was made by HPLC-u.v. calibrated with E_2.

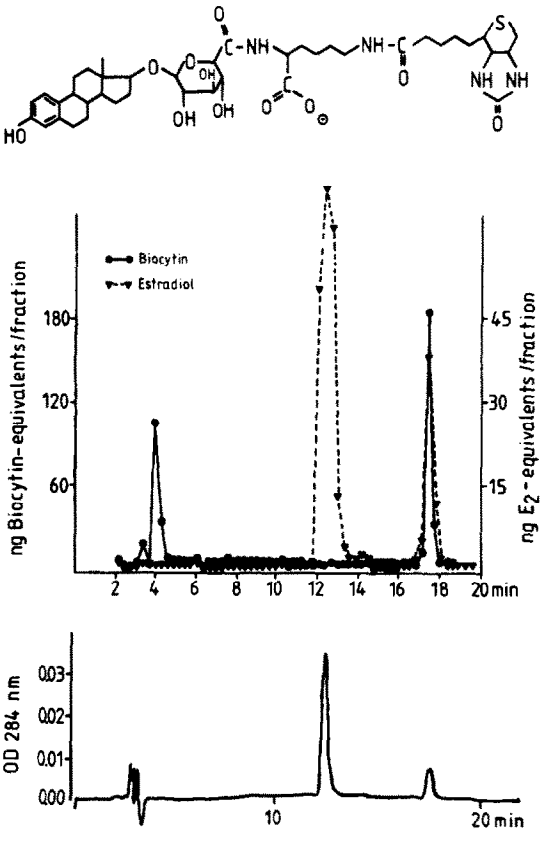

Fig. 1. The predicted chemical structure of biocytinyl-E_2 and its separation from biocytin (4–5 min) and estradiol glucuronide (12–13 min) by HPLC (biocytinyl-E_2 at 17–18 min) as analysed by EIA for E_2, a competitive ligand binding test for biocytin and by u.v.-detection.

The binding activities of the antibodies used in IAC and EIA are summarized in Table 1. From these data it is obvious that estrone and estradiol-17α must be separated during IAC, because the EIA would detect these compounds. Figure 2 (upper panel) demonstrates that estrone in fact binds to the anti-E_2-gel, but it could be eluted prior to E_2 by the described conditions. The other steroid hormones presented showed only very minor retention on the gel. The capacity was tested by addition of E_2 to constant [^{3}H]E_2 (Fig. 2–lower panel). The binding capacity was sufficient up to about 0.5 ng, which is within the normal physiological range of ruminants. If higher E_2-levels were expected, reduced volumes (<0.5 ml) were applied to the column. Under these conditions the mean recovery of [^{3}H]E_2 amounted to 69 + 4.6% over the whole purification process including extraction from plasma.

The sensitivity of the new EIA (Fig. 3) at 50% relative binding was 2.5 times better in comparison to our former EIA using directly linked alkaline phosphatase as a label [6] and 10 times better than our RIA with [^{3}H]E_2 [9]. Also the amount of antibody needed was reduced. Absolute sensitivity was 0.12 pg/well at 94% relative binding. Combining IAC and EIA a large excess of other physiological estro-

Table 1. Crossreactivities of E_2-antibodies

	IAC[a]	EIA
Antigen	E_2-6-CMO-BSA	E_2-17-HS-BSA
Estradiol-17β	100%	100%
Estrone	0.7%	100%
Estradiol-17α	0.9%	66%
Ethinylestradiol	—	14%
Estriol	<0.1%	1.5%
Estradiol-3-benzoate	50%	<0.1%
Trenbolone	1%	<0.1%
19-Nortestosterone	0.5%	0.1%
Progesterone	—	<0.1%
Testosterone	<0.25%	<0.1%
Zeranol	<0.25%	<0.1%
DES	<0.25%	<0.1%

[a]Data were generated in a RIA-system.

gens and normal levels of other steroid hormones were completely discriminated during the test system (Table 2). The interassay variation of the IAC/EIA was determined and there was a clear dependence on the E_2-level in plasma (Table 3). The intrassay coefficient of variation at 5.1 pg E_2/ml amounted to

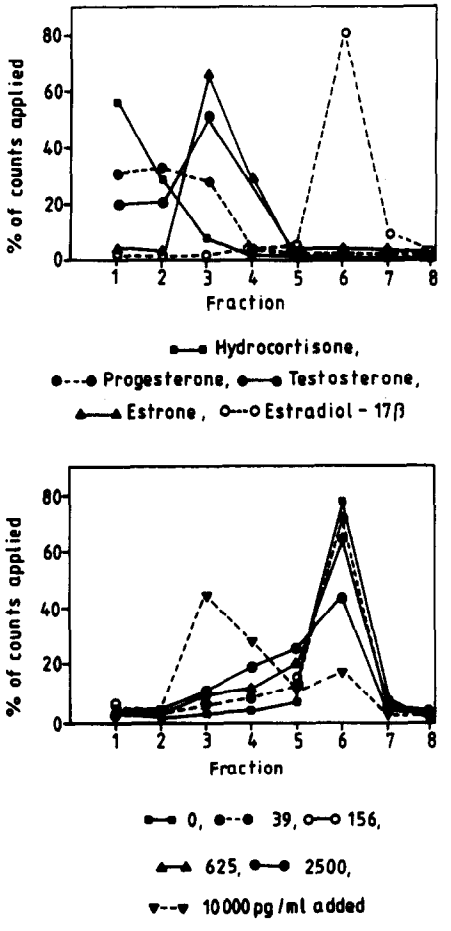

Fig. 2. Retention of different [^{3}H]steroids (upper panel), capacity for estradiol-17β (lower panel) during immunoaffinity chromatography on 200 μg anti-E_2-Sepharose gel. Fraction 1: effluent; Fraction 2: 1 ml phosphate buffer; Fraction 3–5: 1 ml 40% methanol each; fraction 6 and 7: 0.8 ml 80% methanol each; Fraction 8: 1 ml 20% methanol.

Table 2. Recoveries of various steroids by the IAC/EIA for E_2

Type of steroid added	Amount added in plasma (pg/ml)	Amount assayed for E_2 (pg/ml)
None	—	3.5
Estrone	100	3.8
Estradiol-17α	100	4.1
Estriol	100	3.7
Testosterone	10,000	4.9
Progesterone	10,000	4.4
Hydrocortisone	10,000	3.6

Table 3. The precision and interassay coefficient of variation (CV) of the IAC/EIA

E_2 added in plasma (pg/ml)	Amount assayed (pg/ml)	CV (%)	Number assayed
None	3.0	17.4	21
3.13	5.2	16.3	21
6.25	8.5	15.1	11
12.5	16.5	14.0	11
25	30.9	12.5	11
50	57.1	12.2	11
100	110.3	9.1	11

10.3% (n = 16). Also precision is presented in Table 3 and linear regression of added versus assayed E_2-levels was calculated: $R^2 = 0.99$, slope = 1.08. The practical detection limit in plasma depended on the quality of solvents used during the purification. Only with fresh, clean solvents undetectable procedural blanks were obtained and only under these conditions down to 0.5 pg E_2/ml could be measured.

The IAC/EIA was used for samples from untreated animals and for veal calves after E_2-treatment (Table 4). Figure 4 presents peripheral levels of estradiol and progesterone during the estrous cycle; the results confirm that during the luteal phase E_2-levels are varying around 1 pg/ml and even during the estrous only up to 7 pg/ml were found. There are much higher levels during pregnancy and also in bulls up to 30 pg E_2/ml were present.

In plasma samples from untreated calves only up to 2.7 pg E_2/ml was measured and there were similar concentrations in plasma from 10 animals after illegal treatment with estradiol-benzoate and unknown waiting period. Much higher levels up to 1100 pg

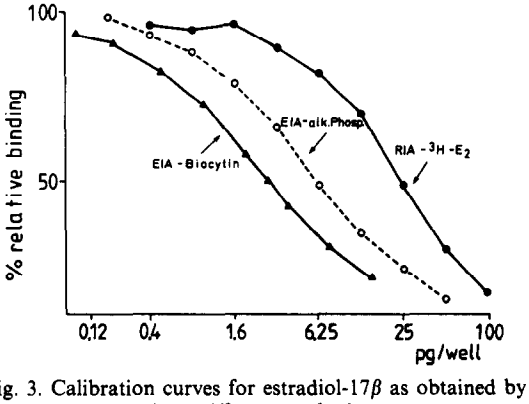

Fig. 3. Calibration curves for estradiol-17β as obtained by three different methods.

Table 4. Ranges of estradiol-17β levels in plasma of cattle as measured by IAC/EIA

Type of animal	Number of animals	Range (pg/ml)
Calves < 6 month; untreated	16 ♀	0.5–2.7
Calves < 6 month; untreated	10 ♂	0.5–2.6
Cycling cows	2	0.5–7
Cows; last month of pregnancy	7	9–310
Mature bulls > 2 yr	3	5–30
Treated calves; silicone rubber implant with 45 mg E_2, day 7	5 ♀ 5 ♂	0.8–84
Treated calf; illegal cocktail, 100 mg E_2-benzoate, first 21 days	1	6–1110
Calves; treated illegally, unknown waiting period	5 ♀ 5 ♂	0.5–2.4

E_2/ml were attained during the beginning of a model experiment with a calf, that had been injected intramuscularly with 4 ml of an illicit anabolic "cocktail". Levels declined to 6 pg/ml after three weeks, which was hardly different from controls (Fig. 5). Total steroidal estrogens followed a similar pattern in this treated animal at 5 times higher level. Silicone rubber implants caused less elevated concentrations being similar in males (26.3 ± 11.2; mean $\pm$ SEM) and females (29.8 ± 10.4).

DISCUSSION

For valid estimation of very low E_2-levels in plasma of ruminants an efficient prepurification system and a very sensitive assay is essential. For prepurification liquid/liquid-partitions or different chromatographies (HPLC, TLC) are classical methods. The elaborated immunoaffinity chromatography represents a much simpler, very selective method that requires minimal equipment and materials. The samples for a whole microtitre plate can be processed simultaneously within 2–3 h. The initial experiments showed that the capacity of the gel and binding to other steroids with a lower affinity needed to be investigated. Testosterone and progesterone show negligable crossreactivity during RIA with the E_2-6-CMO antibody (Table 1), but during IAC there is considerable retention by this antibody. However, these steroids were washed out prior to selective elution of E_2, as documented with [^{3}H]steroids and by IAC/EIA. The IAC-procedure was also possible with unextracted plasma, if the columns were used only one or two times, but much more anti-E_2-gel was needed. After plasma extraction the gel could be used at least ten times. Similar IAC-methods have been used earlier for clean-up prior to GC/MS [10–12].

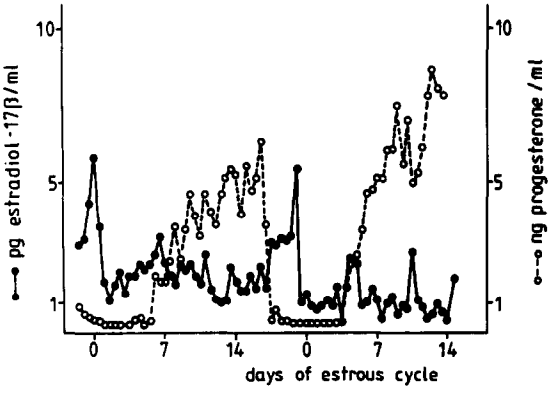

Fig. 4. Estradiol-17β (IAC/EIA) and progesterone (EIA) in blood plasma from the aorta of a cycling cow (Brown Swiss).

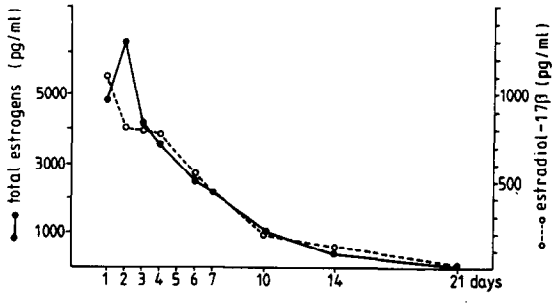

Fig. 5. Total estrogens (hydrolysis/EIA) and estradiol-17β (IAC/EIA) in blood plasma from the jugular vein of a calf after intramuscular injection of 4 ml illicit "cocktail" containing 25 mg estradiol-benzoate/ml.

The synthesis of biocytinyl-E_2 is not very complicated and only a stable peptide bond is formed. The classical route of biotinylation with *N*-hydroxysuccinimido-biotin would need the introduction of an amino-group on the steroid, which should be a more complicated process. In addition the bridge formed is quite hydrophilic due to presence of the free carboxy group of L-lysine and the sugar moity, making the product water soluble. The glucuronide also provides the bridge heterology between the tracer (Biocytinyl-E_2) and the antigen (E_2-17-HS-BSA) used for immunization, which is desirable for a sensitive test and steep calibration curves. The same bridge system was used in our former EIA that uses directly linked alkaline phosphatase as a tracer [7]. With the new system, a better sensitivity was obtained. This cannot be explained by the different enzymes used, because alkaline phosphatase and horseradish peroxidase give a similar sensitivity of the EIA when linked directly to the steroid [13]. The attained sensitivities have not yet been described for EIAs with simple photometric detection and even EIAs with fluorometric [5, 14] or luminescence detection [14, 15] did not provide better sensitivities.

In most EIAs, the enzyme is incubated together with the sample, which may result in a partial inactivation of the enzyme by factors from the plasma, solvents or other chemicals from the samples [16]; this

is especially problematic during control for anabolic agents, because very different matrices have to be analysed (e.g. faeces, urine, muscle extracts) and steroids are measured in solvent mixtures resulting from HPLC or liquid/liquid-partitions. In our assay, only biocytinyl-E_2 comes in contact with the sample constituents and the sample is removed prior to the short incubation with the enzyme. Prior to the description of the "general assay protocol" most steps needed to be optimized intensively for optimal times, temperatures and ligand concentrations. All conditions were chosen in a manner that equilibrium or saturation of binding was achieved in order to get high reproducibilities. The quality of the streptavidin-peroxidase is of particular importance; good optical densities ($O.D._{max} = 1.0$–1.2) were only attained if more than 1 mol of peroxidase was bound per mol of streptavidin.

The application of the combined method IAC/EIA confirms that E_2 is regulated at a very low level in bovines and only up to 7 pg E_2/ml plasma are present during estrous. Earlier studies document that a mean level of 5–10 pg E_2/ml is optimal also for maximal growth promotion in steers [17]; such a level can be achieved up to 400 days after implantation of a silicone rubber implant with incorporated cristalline estradiol. Under these conditions residues in edible tissues are similar to plasma levels [18]. E_2-levels are slightly elevated in plasma from our smaller veal calves treated with the same implant. Illegal mixtures from the black market have different undefined pharmacokinetical behaviour; the investigated cocktail caused an initial rise in E_2-levels of a hundred times more than required, but its anabolic effect was limited to about three weeks due to rapid decrease of peripheral E_2-levels. Normal E_2-levels in plasma therefore do not prove that injection sites are also depleted from steroid residues, because estradiol benzoate has been demonstrated in carcases of those calves suspected for illegal treatment [19], and with normal plasma E_2-levels in this study (Table 1). Nevertheless, the estimation of E_2 in plasma may be a useful parameter for controlling illegal applications of E_2 or its esters and our preliminary studies indicate that levels above 10 pg E_2/ml in calves point to a exogenous E_2-source. But for commitment of a threshold value more intense studies and a validating method for forensic purposes are necessary.

Beside those applications described here there may be further application for the new methods also for other purposes (e.g. sensitive E_2 estimation in small volumes of cell culture or perfusion media). Both methods—IAC and biotin-streptavidin amplified EIA–could be a model for determination of other steroids in the future.

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