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Detection of Anabolic Residues in Misplaced Implantation Sites in Cattle

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Eight weeks before slaughter, 26 heifers, 2 calves, and 1 steer were implanted with licensed anabolic preparations at off-label injection sites. After slaughter, 24 of 31 implantation sites (77%) were detected. Residual pellets of Revalor H contained a mean of 42.9 mg trenbolone acetate (range 19.8–57.7 mg) and 4.6 mg (1.96–6.45 mg) estradiol, corresponding to 30% (19.8–57.7%) and 32.7% (14.0–46.6%) of the originally applied dose, respectively. In the tissue areas containing residual Revalor H pellets, total residues ranged from 14.8 μg to 12.6 mg trenbolone acetate, 41.7 μg to 1.45 mg trenbolone, and 11.1 μg to 3.39 mg estradiol. The outer tissue areas of the injection sites contained <2 μg hormones. The preparations Synovex H, Finaplix H, Implus S, and Component EC behaved similarly to Revalor H. Residues of Synovex Plus were low, whereas the Compudose silicone rubber contained 58.8% of the implanted dose, but left no significant tissue residues. If implantation sites are processed in meat manufacturing, international threshold levels of the respective substances will be exceeded in tons of meat products.

Anabolic sex hormones have been used as growth-promoting agents in farm animals for 4 decades. After the license of preparations containing the naturally occurring gestagen progesterone, the estrogen estradiol, or the androgen testosterone (or their esters) for use in cattle in the United States during the 1950s, xenobiotic substances were also registered. In 1968, the synthetic gestagen melengestrol acetate (MGA) was approved as a feed additive to increase weight gain in heifers; this was followed 1 year later by approval of the estrogenic substance zeranol, a nonsteroid resorcylic acid lactone derived from the fusarium toxin zearalenone. In 1986, the U.S. Food and Drug Administration (FDA) licensed trenbolone acetate, which is superior to the physiological hormone testosterone in terms of quantitative effectiveness. Its strong anabolizing ability contributes to its androgenic potential and to its antiglucocorticoid (1) and gestagen (2) side-effects. The conditions of approval were subject to compliance with Good Veterinary Practice (GVP), which, for anabolic agents, implies (*1*) that each hormone (pellet or feedstuff) is administered to animals in accordance with the manufacturer's instructions, which follow the legislation of the respective country; and (*2*) that each hormone injection site is discarded during slaughter, and that implants will not enter human food or animal feed.

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The substances (except MGA) are licensed as implant preparations that must be injected hypodermically into the pinna of the ear. The implanted ear must be discarded after slaughter to avoid transition into the human or animal food chain. The use of implants is also restricted by the kind of animal, e.g., progesterone is not licensed for use in heifers, and testosterone propionate is not licensed for steers. In calves, testosterone and trenbolone are prohibited.

It was assumed that in countries having licensed anabolic agents abuse of implants (misplacement, overdosing by multiple application of implants, usage in nonauthorized types of animals or species) would be of minor interest, because legal measures are available. However, illegal injection of trenbolone in calves has been documented in Canada (3). The extraordinarily high residue levels could hardly be explained by correct injection of licensed steer- or heifer-preparations into the ears of calves. In a study sponsored by the European Commission, imported beef from the Hormone Free Cattle (HFC) program was monitored. The HFC program was undertaken by the U.S. Department of Agriculture together with beef producers, and enabled export of a specified amount of beef to the European Union (EU), where an import ban prohibits marketing of regular beef from the United States and Canada. Results of that study (4–6) showed that 12% of 258 meat and liver samples were positive for MGA, trenbolone, or zeranol. In addition, DNA gender identification showed that 3 of the 19 meat samples containing MGA originated from male animals, even though treatment of male animals with MGA is not approved in the United States. Of 38 samples of calf meat, 7 contained trenbolone, and 4 of 7 samples of calf liver contained trenbolone alone or trenbolone + zeranol. These substances violate the HFC-status and are not licensed for use in calves.

Hence, compliance with GVP cannot be assumed. Even implantation into animal parts other than the ear cannot be ex-

cluded because inadequate immobilization of the animal to gain unimpaired access to the ear might result in unintentional implantation in another site. Such an injection into an off-label site could severely impair food safety.

The present study examines potential misuse via off-label implantation and provides accurate data on residues of anabolic implant preparations in misplaced injection sites. These residues and anabolic preparations are monitored by liquid chromatography (LC) with diode array detection (DAD) as previously proposed (7–9).

Experimental

Animal Experiments

Five Holstein-Friesian heifers were each injected in the neck with a single dose of Revalor H 8 weeks before slaughter. The exact site of injection was in the middle between shoulder and head, about 6 cm below the cervical spine. These basic conditions were modified in later experiments by variations in injection site, preparation, application time, and sexual status of the animal (Table 1). To ensure precise injection, animals were confined in a head gate with their heads completely fixed to the lattice by ropes. All preparations were injected with original implanter pistols according to the manufacturer's instructions, except for the application site. After the prescribed application time, the animals were slaughtered at an EU-official slaughterhouse.

At the time of hormone treatment, the heifers were 77–99 weeks old with an average weight of 367 kg. The steer and the male and female calves weighed 389, 140, and 105 kg at age 102, 13, and 14 weeks, respectively. The animals were fed 2 kg hay per day and head and corn silage ad libidum. All animals were acquired from an approved livestock provider; a veterinary inspection ensured their health condition. The heifers were cycling and gynecologically intact.

Initial Blood Sampling

An initial blood sample was taken to screen for previous hormonal treatment. The plasma concentrations of trenbolone, zeranol, and MGA were measured by standardized enzyme immunoassay (EIA) kits (R-Biopharm, Darmstadt, Germany) described previously (10, 11). Residues of the substances were below the detection limits of 43, 113, and 22 pg/mL, respectively. Plasma concentrations of progesterone, also determined by EIA (12), corresponded to the estrus cycle. Results of the initial blood screening and the gynecologic investigation did not indicate previous use of anabolic sex hormones.

Preparations, Standards, and Reagents

The implant preparations used in this study are commercially available in the United States. Table 2 summarizes the properties of the products. The content was verified by LC–DAD, and identity of the active ingredients was confirmed by gas chromatography with mass spectrometric detection (GC/MS).

All standard material was purchased from ICN Biomedicals (Costa Mesa, CA; 16 α-methylprogesterone), Steraloids (Newport, RI; D-equilenin), or Sigma Chemical Co., (St. Louis, MO; all others). Standard identity was also confirmed by GC/MS, and purity was demonstrated by LC–DAD. All reagents were analytical grade and all solvents were of LC purity.

Residue Analysis

During slaughter, the entire misplaced implantation site, including surrounding tissues (1.5–3 kg), was excised from the carcass. At the laboratory, the injection site was prepared. The residue analysis refers to the visible remainder of the pellets (or silicone rubber), the inner tissue area that contains the injected pellets, and the outer area that surrounds the inner area. The residue analysis was performed by LC–DAD after liquid extraction and solid-phase cleanup. For the inner and outer areas, internal standardization was applied.

Preparation of Implantation Sites

The tissue that was collected at the slaughterhouse and contained the implants was systematically cut into small pieces with a maximum diameter of 5 mm. All detected implants from each site were collected in a single vial and analyzed as a whole. For preparation of the inner area that contained the implants, all tissue within ca 2.5 cm around the pellets was collected. The region surrounding the inner area (i.e., the next 1.5 cm of tissue attached to the inner area) was sampled as the outer area. To prevent contamination of the tissue samples by residual pellets, disposable scalpels were used to extract the implants. Thus, visible residues of the implanted preparations and about 50–150 g of both inner and outer tissue were collected. An exception was made at the external ear, where a 3 cm area of the pinna of the ear surrounding the injection site (including cartilage and skin) served as the inner area and the earground was the outer area. At the crown, no tissue was extractable because the pellets were adhering to the bone.

Extraction and Cleanup

(**a**) *Inner and outer tissue areas.*—Samples were coarsely crushed into small pieces and homogenized on a Miccra (Art moderne Labortechnik, Mühlheim, Germany) high-speed mixer system together with an equal amount of 67mM phosphate buffer, pH 7.2 (the pinna of the ear sample required twice the amount of buffer). After addition of internal standard, 8 g homogenized sample was extracted twice by *tert* butyl-methyl-ether (TBME) with agitation overnight. The extraction vials were centrifuged (850 × *g*, 15 min, 4°C) and frozen (–60°C). The extract was then decanted and evaporated. The residue was redissolved in 2 mL methanol, thoroughly shaken, and frozen (–60°C). After centrifugation (850 × *g*, 2 min, –10°C), the supernatant was diluted with 4.5 mL water. The solution was applied to an octadecyl-silica cartridge, which was washed twice with 1 mL (40%) methanol. The hormones were eluted with 1.5 mL methanol. The metha-

Table 1. Animal experiments

Study	Animal No.	Age (sex)	Preparation	Dose	Duration (days)	Application site (area of implantation)
I. Basic	05804	Heifer	Revalor H	1	56	Neck (*M. splenius, M. serratus*)
	07992					
	05801					
	05811					
	07998					
II. Different locations	07990	Heifer	Revalor H	1	56	Shoulder (*M. deltoides*)
	07984					Leg (*M. semitendinosus*)
	07981					External ear (labeled location)
	07980					Earground a (*M. cervicoauricolaris*)
	07999					Earground b (*M. cervicoscutularis*)
	07977					Earground c (*M. cervicoauricolaris*)
	05805					Base of the tail (*M. coccygeus*)
	05810					Breast muscle (*M. pectoralis desc.*)
	07986					Dewlap
	07991					Neck, subcutaneous
	07993					Scutulum
	07995					Crown (*M. frontalis*)
III. Different preparations	07996	Heifer	Finaplix H	1	56	Neck (*M. splenius, M. serratus*)
	08000		Synovex Plus			
	07975		Synovex H			
	07994		Compudose			
	07982		Component EC			
	07985		Implus S			
	07983		Ralgro			
IV. Different duration	05809	Heifer	Revalor H	1	84	Neck (*M. splenius, M. serratus*)
	07978			1	28	
	24901			3	28, 84, 140	
V. Different sex/age	67010	Steer	Revalor H	1	56	Neck (*M. splenius, M. serratus*)
	89922	Calf (m)				
	26521	Calf (f)				
Control	07987	Heifer	Control	0	. . .	(No injection)
	70998					

nol was evaporated and the residue was redissolved in acetonitrile for LC analysis.

(**b**) *Implants*.—After addition of 8 mL water, the pellets (original and recovered) were extracted 3 times with 8 mL TBME according to the tissue samples. After evaporation of the combined ether phases, the residue was dissolved in 10.0 mL methanol. Aliquots (100 μL) of the solution were evaporated and redissolved in LC solvent.

Quantification of Residues by LC–DAD

The LC analysis was performed on a Beckman (Munich, Germany) LC system with a 250 × 4.0 mm reversed-phase column (Prontosil 120-5-C_{18} H, 5.0 μm; Bischoff, Leonberg, Germany). The mobile phase was acetonitrile–20mM tris–acetate buffer, pH 7.2, (42 + 58, v/v, solvent A; or 70 + 30, v/v, solvent B). The separation was performed by running solvent A 20 min, and solvent B 19 min, followed by cleaning with 97% (v/v) acetonitrile (5 min) and equilibration with solvent A (10 min), all at a flow rate of 1 mL/min. Column temperature was set at 25°C. The system was calibrated by integration of the peak areas and a regression line ($R^2 > 0.999$) through 6 calibration points covering at least 1 decimal place. The peaks were identified by retention times and UV spectra. Tissue analyses were standardized by internal standard added to the sample before extraction, and correction of the measured analyte concentration by the fraction of recovered internal standard. Internal standard substances were testosterone acetate (for trenbolone acetate, estradiol benzoate, and testos-

Table 2. Properties of the preparations and recovery rates of hormone quantification

Trade name	Manufacturer	Characteristics, single dose	Active principle	Labeled content,[a] mg	Measured content, mg; mean ± SD; $n = 3$	Recovery, %
Revalor H	Hoechst Rousell Vet, Sommerville, NJ	7 Yellow pellets	Trenbolone acetate	140	146.04 ± 14.95	104.31
			Estradiol	14	13.37 ± 0.98	95.52
Finaplix H	Hoechst Rousell Vet, Sommerville, NJ	10 White pellets	Trenbolone acetate	200	188.45 ± 39.48	94.22
Synovex Plus	Fort Dodge Laboratories, Fort Dodge, IA	8 Lightly red pellets	Trenbolone acetate	200	201.08 ± 25.56	100.54
			Estradiol–benzoate	28	24.89 ± 1.63	88.90
Synovex H	Fort Dodge Laboratories, Fort Dodge, IA	8 White pellets	Testosterone propionate	200	189.20 ± 22.14	94.60
			Estradiol–benzoate	20	18.85 ± 2.13	94.24
Compudose	Vetlife, Inc., Norcross, GA	1 Slightly yellow silicone rubber	Estradiol	25.7	24.91 ± 0.60	96.93
Component EC	Vetlife, Inc., Norcross, GA	4 Orange pellets	Pogesterone	100	83.60 ± 8.61	83.60
			Estradiol–benzoate	10	8.54 ± 0.87	85.35
Implus-S	Upjohn, Kalamazoo, MI	8 Orange pellets	Progesterone	200	167.23 ± 5.72	83.62
			Estradiol–benzoate	20	18.08 ± 1.55	90.40
Ralgro	Mallinckrodt Veterinary, Inc., Mundelein, IL	3 White pellets	Zeranol	36	32.05 ± 1.84	89.02

[a] According to the statements of the manufacturers.

terone propionate); D-equilenine (d-1,3,5(19),6,8-estrapentane-3-ol-17-one; for trenbolone, estradiol, testosterone, and zeranol); and 16 α-methylprogesterone (for progesterone). Table 3 lists the retention times of the substances.

Method Validation

(**a**) *Accuracy*.—Accuracy was determined as recovery from fortified (spiked) blank sample material collected from the necks of control animals. All recoveries were determined for 3 different spike values (high, middle, and low expected hormone concentrations in inner and outer areas of the implantation site) in 5 (progesterone, zeranol) or 10 replicates (others) each. Table 4 shows absolute recoveries and recoveries after correction by the internal standard for analytes. The standard substances had mean (n = 10) absolute recoveries of 68.1% (range 52.9–66.3%; D-equilenine), 62.1% (56.0–65.2%; 16 α-methylprogesterone), and 63.1% (60.3–66.7%; testosterone acetate).

(**b**) *Precision*.—Precision is the closeness of agreement between results obtained by applying experimental procedure several times; it covers repeatability and reproducibility. This study validated repeatability conditions (i.e., analysis of identical test material in the same laboratory by the same operator using the same equipment within short intervals of time), because it was not necessary to perform the method in other laboratories with other equipment. Precision is calculated as the coefficient of variation (% CV) of the 10 (5) results (after standardization) obtained from accuracy experiments. The last column of Table 4 contains the coefficients of variation.

(**c**) *Limits of determination, quantitation, and detection*.—The smallest analyte for which the method was validated with the specified accuracy and precision is defined as the limit of determination (Table 4). However, smaller concentrations to a limit of 10 ng/g could be quantitated, depending on the appearance of the chromatogram.

Residue Identity Checks by GC/MS

Analyte identity checks for all implants were performed by GC/MS of heptafluorobutyryl derivatives based on procedures described earlier (13, 14). The possibility of detecting steroid esters by GC/MS without hydrolysis was recently shown for melengestrol acetate (11).

For at least one of each kind of recovered implants, a purified aliquot (5 μL) of extract solution was diluted with 50 μL acetone, and derivatized with 20 μL heptafluorobutyric anhydride (HFBA). After evaporation, the sample was redissolved in 100 μL octane and 1 μL was injected into the GC/MS (Platform II, Micromass, Manchester, UK). Identification was based on specific retention times and mass spectra [electron impact ionization (EI), *m/z* 300–700]. The characteristic ions used for identification are presented in Table 5.

Quality Assurance

A Quality Assurance System guaranteed the accuracy of the entire study. All laboratory procedures followed a Quality Assurance Manual. The status of Quality Assurance/Quality

Table 3. Retention times during LC quantification

Substance	Retention time, min
Trenbolone	8.3
Zeranol	10.7
Estradiol-17β	11.6
Estradiol-17α	13.8
D-Equilenine	14.8
Estrone	18.1
Trenbolone acetate	26.3
Progesterone	27.2
16α-Methylprogesterone	30
Testosterone acetate	30.3
Estradiol benzoate	37.6
Testosterone propionate	36.1

Management was inspected by the European Community Reference Laboratory (CRL, Bilthoven, The Netherlands).

Results

Extraction of Residual Pellets from Implantation Sites

One complete dose of the implant preparations used in these experiments consisted of either 4, 7, 8, or 10 single pellets in a line (except Compudose, which is a single silicone rubber). Altogether, 31 implantations were performed, omitting implantation in the ear of animal 07981. Twenty-four application sites (77%) were detected by the presence of residual pellets. The number of recovered pellets was smaller (67%) because not all of the pellets of a respective preparation could be found. Table 6 lists the results of pellet extraction by different parts of the experiment. Individual numbers of recovered pellets for each animal are included in Table 7.

The recovered pellets were predominantly embedded in connective tissue sheaths. Generally, the size of the pellets was smaller than that of the original, but sometimes the external shape was nearly intact. One pellet, located next to nearly intact pellets, was completely fluidized, but clearly detectable in a tissue sheath, i.e., the condition of the implant or the implanted pellets, respectively, varied between obviously intact and presumably dissolved completely. At some implantation sites, single pellets were found within areas of 3–4 cm in diameter, indicating pellet migration within the off-label implantation sites of that experiment.

Hormone Residues in Detected Pellets

Basic experiment: Revalor H.—After a single misplaced application of Revalor H 8 weeks before slaughter, the average amounts of parent compounds detectable in recovered pellets were 42.9 mg (range 19.8–57.5 mg) for trenbolone acetate and 4.6 mg (range 1.96–6.45 mg) for estradiol (n = 14). That finding corresponds to 30.0% (14.1–41.1) and 32.7% (14.0–46.6) recovery of the applied dose. Calculated recovery rates were even higher when detected residues were compared with the dose of hormones originally present in the recovered pellets. The similar range of residues after injection in necks and other misplacement sites is outstanding, i.e., the hormone residues are predominantly dependent on the number of pellets recovered. Application in the authorized location (pinna of the ear) resulted in clearly higher hormone residues of Revalor H (74.3 and 7.91 mg, corresponding to recoveries over 50%; Table 7).

Variation in gender, age, and duration.—The use of Revalor H in calves (which is not licensed in the United States) causes residues similar to those found in heifers. For the steer, no statement is possible, because no pellets were found. When the implant was injected 4 weeks before slaughter, the recovery of mother compounds was higher [59.0 and 60.6 mg (or %) of the maximum dose in the pellets]. In the experiment with 3 applications (4, 12, and 20 weeks before slaughter), an assignment of detected pellets to certain applications was not possible in the carcass. It is presumed that after longer application periods the residues decrease.

Variation of the preparation.—Compudose is striking for its high recovery rate (15.0 mg of 25.7 mg estradiol), and Synovex Plus for its low recovery rate (6.3 and 6.5% in the detected pellets). However, because those were single observations, they must be regarded as exemplary. Synovex H, Finaplix H, Implus S, and Component EC behaved in a manner similar to Revalor H. Ralgro was not evaluated because the pellets were either completely dissolved or it was not possible to find them.

Hydrolysis of esterified steroids.—In the recovered pellets, a small fraction of trenbolone acetate was hydrolyzed to trenbolone-17β. The absolute residues of free trenbolone in all implantation sites of trenbolone acetate ranged from 0.01 to 0.12 mg (mean 0.06 mg, n = 21), corresponding to a fraction between 0.1 to 0.3% (mean 0.15%), with no distinctive difference between the preparations (Revalor H, Finaplix H, Synovex Plus) and different applications. Hydrolysis of estradiol benzoate and of testosterone propionate was not observed in the respective pellets.

Residues in Inner Areas around the Pellets

(**a**) *Revalor H.*—After application of Revalor H, residues of trenbolone acetate, trenbolone-17β, and estradiol were widely scattered in the inner areas around the recovered pellets. Trenbolone acetate ranged from 0.21 to 118 μg/g, trenbolone-17β from 0.58 to 19.0 μg/g, and estradiol from 11.1 to 3931 μg/g. Multiplication of the concentrations by the total sample masses resulted in the corresponding total amounts of residues, covering a factor of 1000. Trenbolone acetate residues were between 14.8 μg and 12.56 mg, trenbolone-17β between 41.7 μg and 1.45 mg, and estradiol between 11.1 μg and 3.93 mg. Values in the mg range seem to be outliers and can be easily explained: Before preparation of the implantation sites, the collected tissue area was intact and the implants were not visible. In order to locate the implants, the surrounding tissue had to be trenched. During the cutting,

Table 4. Accuracy and precision of the determination of hormone residues in implantation sites by LC–DAD

Hormone	Spike (ng/g meat)	Absolute recovery, mean, %	Recovery after standardization, mean, %	Recovery after standardization, range, %	Precision (repeatability) CV, %
Trenbolone	1400	57.4	92.2	87.3–96.6	3.5
	352	58.5	92.6	90.4–96.3	2.0
	99.1	62.7	102.9	99.2–110.0	3.2
Zeranol	2630	69.4	104.1	103.7–104.2	0.25
	665	71.3	102.8	96.4–106.0	3.6
	124	73.5	108.0	100.5–122.6	8.1
Estradiol-17β	1800	66.6	107.0	103.1–110.1	1.9
	451	67.4	106.6	103.7–107.9	2.4
	127	61.4	100.7	92.5–117.9	7.5
Testosterone	1510	54.9	88.3	85.7–90.8	2.3
	378	55.8	88.4	86.4–91.6	1.9
	106	56.5	92.6	86.4–97.3	3.8
Trenbolone acetate	10900	62.7	98.7	96.0–102.4	1.8
	2630	63.4	101.7	94.6–107.4	4.8
	886	66.1	104.4	97.3–111.0	4.1
Progesterone	4610	61.0	98.7	97.3–101.3	1.8
	1550	61.6	100.5	99.2–101.3	0.84
	460	68.4	108.2	105.0–109.7	1.7
Estradiol benzoate	9640	60.0	94.3	91.1–96.8	1.8
	2330	59.9	96.0	87.2–103.9	5.5
	780	62.4	98.6	93.1–103.4	3.3
Testosterone propionate	11400	61.5	96.7	94.2–99.1	1.4
	2760	61.1	97.9	90.6–103.7	5.0
	926	61.7	97.3	91.6–103.3	3.9

contacting of unknown pellets with the knife or scalpel could not be prevented. Thus, the surrounding tissue may have been contaminated by particles of the preparations. Compared with the residual pellets, relatively high amounts of hydrolyzed, free trenbolone-17β were apparent. When the sum of trenbolone acetate and trenbolone-17β is set as 100%, between 7.4 and 83% (mean of 36%) of the original substance was hydrolyzed (Table 8).

(**b**) *Other preparations*.—In single observations, residue amounts of Finaplix H, Synovex H, Component EC, and Implus S were similar to those of Revalor H in the enclosing tissues. Marked degrees of hydrolysis of trenbolone acetate for Synovex Plus (84%), of testosterone propionate for Synovex H (17%), and of estradiol benzoate for Component EC and Implus S (8 and 11%) were apparent. Although the residual estradiol in the silicone rubber Compudose was relatively high (58.4%), the preparation did not cause particular estradiol levels in the rubber surrounding tissue. Synovex Plus was outstanding for its low residues. The zeranol implantation site could not be found (similar to 6 of 24 Revalor H implantations) and therefore cannot be evaluated.

Residues in Outer Areas around the Pellets

(**a**) *Revalor H*.—The outer areas of Revalor H implantation sites contained <2 μg trenbolone acetate (data not shown). Exceptions were represented by neck (07998; 5.3 μg), earground (07977; 20.3 μg), breast muscle (05810; 8.9 μg), and the implantation 28 rather than 56 days before slaughter (05809; 58.5 μg). Outlying levels might originate from inevitable contamination during preparation. Tissue concentrations were normally <10 ng/g, but ranged from 50 to 220 ng/g in the exceptions shown above. Free trenbolone-17β was detectable in 15 of the 24 samples, with mean tissue concentrations of 37 ng/g, resulting in mean total amounts of 6 μg. Only 6 of the outer areas of Revalor H implantation sites contained residues of estradiol above the detection limit. When detectable, its concentrations were 140 ng/g and its total amounts were 26 μg as a mean.

Table 5. Characteristic ions (*m/z*) for residue identity checks by GC/MS

Substance	Characteristic ions, *m/z*
Trenbolone acetate	506, 446, 431, 419
Testosterone propionate	540, 525, 466, 451
Progesterone	510, 495, 467, 425
Estradiol	664, 451, 409, 356
Estradiol benzoate	572, 359

(**b**) *Other preparations*.—The outer area of the Finaplix H implantation site was striking for its high residue levels of trenbolone acetate (13.8 μg/g or 677 μg, respectively) and trenbolone-17β (4.5 μg/g or 221 μg, respectively) as compared to its inner area and to other preparations. Synovex Plus caused low residues: 2.5 μg total trenbolone, of which 39% were hydrolyzed to trenbolone-17β, and 2.8 μg to estradiol benzoate. The residues of testosterone propionate (10.8 μg), free testosterone (3.6 μg), and estradiol benzoate (5.6 μg) after Finaplix H application were higher. For Compudose, Component EC, Implus, and Ralgro no hormone residues were detectable in the outer areas.

Discussion

Hormone Residues at Off-Label Implantation Sites

The implant preparations licensed as hormonal growth promoters are sustained release formulations designed for use in the outer ear. Substances injected into off-label sites clear about 8 weeks after application, with 30% (range 14–47%) recovered from the implantation sites. Our results were in agreement with those of absorption experiments from compressed pellets containing trenbolone acetate and estradiol. After injection into the base of the ear of sheep, the site produced a marked increase in plasma concentrations of estradiol and trenbolone even 15 weeks after implantation (15). At 100 and 140 days after application into the external ear, residuals of 6 and 18% trenbolone acetate were found in the injection sites (16). Recoveries of zeranol were 15 and 12% at 65 and 125 days after implantation.

Results obtained from studies with compressed pellets were compared with those using oily suspensions, which might be observed in illegal practice. At 14 days after injection of 44 mg 4-^{14}C-estradiol-17β into the neck muscle of heifers, only about 0.06% of the original radioactivity remained at or near the injection site (17). However, after injection of oily suspensions of nonradioactive compounds, 1–16% 19-nortestosterone (18), <5% estradiol benzoate and 19-nortestosterone (19), and 0.03% estradiol benzoate (20) were detected at injection sites, indicating that the type of preparation strongly affects retention in tissue. Compressed pellets, which were found to have longest efficiencies in the animal, are potentially harmful when ingested.

Hydrolysis of esterified steroids is negligible in residual pellets, but relevant in surrounding tissue areas. This finding confirms our common understanding of the effective mode of implanted steroid esters: as long as they are embedded in the implant they are inert, but split apart after migration into the circulation.

Residues in Meat and Meat Products

Compressed hormonal implant preparations injected into parts of the animal that are not discarded after slaughter can enter human food even after long periods of withdrawal. Eight weeks after implantation at off-label sites, about 75% of the applications can be identified by visible residual pellets. Combined preparations typically contain hormone residues of 50 mg androgen or gestagen plus 4 mg estrogen. The amounts in the tissue areas around the pellets are lower by a factor of 100–1000 and reach 20–600 μg. Total residues in the outer areas are 300 times lower than in the inner regions and do not exceed 2 μg. Consequently, a risk assessment of hormone residues in misplacement sites must focus on the detectable implanted preparations remaining.

International Threshold Levels for Hormone Residues

Residues of the synthetic compounds trenbolone and zeranol were judged by Joint FAO/WHO Expert Committee on Food Additives (JECFA) and adopted by the Codex

Table 6. Detection of implantation sites and pellets during preparation

	Implantation sites			Pellets		
Experiment	Total No.	No. detected	Detected, %	Total No.	No. detected	Detected, %
I. Basic	5	4	80	35	23	66
II. Different locations	11	9	82	77	62	81
III. Different preparations	7	6	86	42	29	69
IV. Different duration	5	3	60	35	14	40
V. Different sex and age	3	2	67	21	13	62
Total	31	24	77	210	141	67

Table 7. Residues of trenbolone acetate (TBA), estradiol (E2), estradiol benzoate (E2B), testosterone propionate (TP), progesterone (P), and zeranol (Z) in recovered pellets

	Components, mg				Maximum, mg		Found, mg		% Applied dose		% in Found pellets		Hydrolyzed, mg
Study	A	B	Animal No.	Pellets found	A	B	A	B	A	B	A	B	A
I.	140 TBA	+ 14 E2	05804	0									
			07992[a]	6	120	12	37.7	4.52	26.9	32.3	31.4	37.7	0.069
			05801	7	140	14	57.5	6.08	41.1	43.4	41.1	43.4	0.117
			05811	5	100	10	39.6	3.96	28.3	28.3	39.6	39.6	0.055
			07998	5	100	10	34.2	4.01	24.4	28.6	34.2	40.1	0.050
II.	140 TBA	+ 14 E2	07990	7	140	14	54.1	6.53	38.6	46.6	38.6	46.6	0.086
			07984	7	140	14	46.7	5.33	33.3	38.1	33.3	38.1	0.050
			07981	7	140	14	74.3	7.91	53.1	56.5	53.1	56.5	0.080
			07980[a]	6	120	12	28.7	3.22	20.5	23.0	23.9	26.9	0.081
			07999	7	140	14	47.6	5.34	34.0	38.1	34.0	38.1	0.053
			07977	6	120	12	42.5	4.44	30.4	31.7	35.5	37.0	0.043
			05805	0									
			05810	3	60	6	19.8	1.96	14.1	14.0	33.0	32.6	0.026
			07986	6	120	12	48.3	5.28	34.5	37.7	40.3	44.0	0.060
			07991	7	140	14	56.5	6.45	40.4	46.1	40.4	46.1	0.070
			07993	0									
			07995	6	120	12	32.5	2.99	23.2	21.4	27.1	24.9	0.072
III.	200 TBA		07996[a]	9	180		33.3		16.7		18.5		0.050
	200 TBA	+ 28 E2B	08000[a]	3	75	10.5	4.76	0.68	2.4	2.4	6.3	6.5	0.010
	200 TP	+ 20 E2B	07975[a]	4	100	10	31.0	3.26	15.5	16.3	31.0	32.6	<0.01
		25.7 E2	07994[a]	1		25.7		15.0		58.4		58.4	
	100 P	+ 10 E2B	07982[a]	4	100	10	32.9	3.82	32.9	38.2	32.9	38.2	
	200 P	+ 20 E2B	07985[a]	8	200	20	52.6	7.10	26.3	35.5	26.3	35.5	
		36 Z	07983	0									
IV.	140 TBA	+ 14 E2	05809	0									
			07978	5	100	10	59.0	6.06	42.2	43.3	59.0	60.6	0.060
			24901 a	7	140	14	41.6	4.64	29.7	33.1	29.7	33.1	0.040
			24901 b	3	60	6	10.1	1.03	7.2	7.4	16.9	17.2	0.010
			24901 c	0									
V.	140 TBA	+ 14 E2	67010	0									
			89922[a]	6	120	12	36.8	3.89	26.3	27.8	30.7	32.4	0.050
			26521[a]	7	140	14	46.5	4.66	33.2	33.3	33.2	33.3	0.070

[a] Identity of compounds checked by GC/MS.

Table 8. Residues of trenbolone acetate (TBA), estradiol (E2), estradiol benzoate (E2B), testosterone propionate (TP), progesterone (P), and zeranol (Z) in inner tissue areas around the implanted preparations. No tissue could be collected from implantation site 07995.

	Component				Main component		Main component (hydrolized)		Minor component	
Study	A	B	Animal No.	Complete mass, g	Concentration, µg/g	Total amount, µg	Concentration, µg/g	Total amount, µg	Concentration, µg/g	Total amount, µg
I.	TBA	+ E2	05804	204.15	<0.01	<0.01	<0.01	<0.01	0.05	11.09
			07992	124.44	0.26	32.31	0.58	72.22	<0.01	<0.01
			05801	106.15	118.30	12557.51	13.63	1447.19	37.03	3931.19
			05811	152.50	64.57	9847.42	6.53	995.74	19.58	2986.29
			07998	93.46	0.72	67.56	1.27	118.39	1.20	112.17
II.	TBA	+ E2	07990	82.93	3.79	314.59	1.18	97.64	5.38	445.77
			07984	125.34	3.21	402.09	1.40	175.57	4.44	556.55
			07981	20.40	11.51	234.90	2.05	41.72	3.49	71.25
			07980	67.86	1.50	102.03	0.90	61.18	2.50	16984
			07999	78.78	6.84	538.99	2.30	181.21	10.29	810.7
			07977	55.15	4.52	249.46	2.14	118.19	6.57	362.35
			05805	204.04	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
			05810	46.79	0.47	22.02	1.16	54.09	1.12	52.34
			07986	91.56	12.12	1109.50	2.48	227.35	6.91	632.44
			07991	20.67	36.33	750.97	18.97	392.16	53.74	1110.86
			07993	73.36	<0.01	<0.01	0.00	0.16	<0.01	<0.01
III.	TBA		07996	119.72	0.31	37.64	0.15	17.60	—[a]	—[a]
	TBA	+ E2B	08000	118.41	0.01	0.67	0.03	3.43	0.04	4.52
	TP	+ E2B	07975	65.76	3.84	252.84	0.76	50.21	1.66	109.35
		E2	07994	158.08	—[a]	—[a]	—[a]	—[a]	<0.01	<0.01
	P	+ E2B	07982	76.72	2.56	196.13	0.20*	15.13[b]	2.17	166.53
	P	+ E2B	07985	68.72	13.53	929.83	0.47*	32.03[b]	3.93	270.2
		Z	07983	130.33	—[a]	—[a]	—[a]	—[a]	<0.01	<0.01
IV.	TBA	+ E2	05809	147.40	0.00	0.28	0.00	0.07	<0.01	<0.01
			07978	104.24	2.61	271.80	2.11	219.57	3.93	409.8
			24901 a	60.56	75.03	4543.84	5.97	361.32	28.30	1714.06
			24901 b	82.99	4.48	371.38	2.24	186.03	6.14	509.74
V.	TBA	+ E2	67010	151.68	<0.01	<0.01	<0.01	<0.01	0.03	4.61
			89922	57.99	3.49	202.35	2.34	135.65	5.09	295.02
			26521	71.01	0.21	14.79	1.01	71.65	0.65	46.13

[a] Not present.
[b] Estradiol.

Table 9. ADI, MRL, and NOEL/LOEL levels of hormones in meat by JECFA/Codex Alimentarius[a]

Substance	ADI, μg/kg body weight	ADI, μg/day; adult 60 kg	MRL, μg/kg	NOEL/LOEL, mg/day
Trenbolone-17β (6)[b]	0.02	1.2	2	—
Zeranol (6)[b]	0.5	30	2	—
Estradiol-17β (7)[c]	0.05	3	Not specified	0.3 (NOEL)
Testosterone (7)[c]	2	120	Not specified	100 (NOEL)
Progesterone (7)[c]	30	1800	Not specified	200 (LOEL)

[a] Abbreviations: ADI = acceptable daily intake; MRL = maximum residue level; LOEL = lowest observed effect level; JECFA = Joint FAO/WHO Expert Committee on Food Addivites.
[b] (6) = Codex Alimentarius (1999).
[c] (7) = JECFA (1999).

Alimentarius in 1995 (21). Based on acceptable daily intake (ADI) levels, maximum residue levels (MRL) were established for their residues in liver and muscle. Estradiol-17β, testosterone, and progesterone were reassessed by JECFA 1999 (22). The full report has not yet been published, but the summary form is available. MRLs were not specified because it was thought that residues of veterinary drugs in animal tissues had a wide margin of safety for consumption in food if the drug was used in accordance with good veterinary practice. Table 9 summarizes the international threshold values.

Contamination of Meat and Meat Products by Implantation Sites

When implant preparations are misplaced or when the ears of legally treated animals are not discarded after slaughter, milligrams of hormone residues potentially enter human nutrition. By consuming complete implantation sites, the consumer ingests hormone amounts that can have acute effects. A key consideration is the fraction of misplaced implants that might pass postmortem food inspection and thus reach the consumer; however, that question is not answered by our experimental data. Because hypothetically misplaced implants are not visible at the surface of carcasses, the risk that they would not be detected during inspection is very high. Meat inspection does not focus on the detection of foreign objects in skeletal muscle, especially in valuable parts of the carcass. During our experiments, we were often unable to find misplaced implants during sample collection; whole tissue regions into which were injected implants had to be dissected completely to find the hormone pellets. Thus, hypothetical misplaced implantation sites may normally pass official meat inspection at the slaughterhouse. Although such implants might be detected during preparation of meat in the kitchen, this is not a reasonable solution to the problem for consumers.

Implantation sites that are industrially processed into meat products contaminate whole batches of the respective foodstuff (minced meat, sausages, etc.). The resulting hormone residues widely exceed established international threshold levels cited in Table 9. The model calculations in Table 10 illustrate the impairment of meat products caused by implantation sites that reach the processing plants. In the described scenario, a typical implantation site of Revalor H is processed during meat production. Typical batch sizes are 30 kg for butcher-made and 600 kg for industrial products. In that instance, trenbolone acetate and trenbolone-17β are not differentiated. The model calculation can easily be adjusted to other conditions, such as other preparations or dosages. Additionally, if implantation sites are discarded but not physically exterminated, they will be rendered for the processing of animal feed. The respective drugs are not destroyed by the temperatures usually used during that

Table 10. Model calculation for hormone contamination of processed meat by implantation sites

Substance	Trenbolone	Estradiol
Exemplary amount of hormone in one recovered implantation site	42 mg (trenbolone acetate)	4.6 mg (estradiol-17β)
Threshold value	MRL (meat) 2 μg/kg (trenbolone-17β)	ADI (estradiol-17β; adult) 3 μg/500 g (1 daily portion)
Amount of meat in which residues are elevated to the threshold value	21 000 kg	767 kg (1533 daily portions)
Threshold excess in processed meat batch		
Butcher-made (30 kg)	700-fold	25-fold
Industrial (600 kg)	35-fold	1.3-fold

process and become measurable even in fed animals (23). Thus, the hormones might be recycled.

Conclusions

The present study emphasizes the importance of GVP. However, because observance of GVP cannot be assumed, regulatory authorities should consider the possibility of misuse of anabolic agents such as injection at off-label sites. Because of the harmful effects of misplaced implantations on food production, correct injection of the preparations must be ensured, if necessary, by means of official surveillance.

Techniques for implant preparation of compressed pellets are over 40 years old. However, because of the enormous economic benefit and the need to preserve natural resources, growth promoting of farm animals cannot simply be rejected. New research is needed to increase meat production with the help of technologies that will eliminate the risk of leaving significant hormone residues in the animal.

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