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DETERMINATION OF HORMONE CONTAMINANTS IN MILK REPLACERS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND IMMUNOASSAY

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SUMMARY

Certain milk replacers were reported to cause 19-nortestosterone (NT)-positive urine samples after feeding them to veal calves. In order to find the possible source of contamination, milk replacers and crude fat and meat meal from homogenized veal calves and commercial crude fat from a rendering plant were analysed for NT plus its metabolites and constituents of illicit 'cocktails' (NT esters, estradiol benzoate and medroxyprogesterone acetate). The steroids were separated using different high-performance liquid chromatographic systems and measured by specific immunoassays. The results show that animal food processed from carcasses of treated animals contains hormone concentrations that may cause positive urine samples in animals fed on such feed.

INTRODUCTION

Certain milk replacers were recently reported to cause 19-nortestosterone (NT)-positive urine [1]. This was the first time that NT-positive urine had been found without injecting veal calves with corresponding anabolic preparations. As a consequence, it became necessary to distinguish between oral and parenteral application of some anabolics. One way was to investigate kidney fat for NT-17β and 19-norandrostenedione (NA), which are markers for the parenteral application of NT and NT esters [2]. Another method was the direct examination of milk replacers in addition to meat meal and crude fat obtained from homogenized veal calves suspected of being injected with illicit 'cocktails'. Milk replacers may be contaminated by simple addition of anabolics, e.g., by persons fattening veal calves or dealing with animal food and by the manufacturer. On the other hand, contamination could result from injected animals which are processed in rendering plants into crude fat and meat meal. Perished animals and parts of them such as necrotic tissues or ears, which may contain residues of anabolic preparations,

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are homogenized and separated into water, crude fat and meat meal. In Germany, rendering-plant fat is extracted mechanically after heating, whereas in the Benelux states solvents such as hexane are used. During extraction, lipophilic hormones such as steroid esters are usually accumulated in the fat fraction. Crude fat from rendering plants is processed into milk replacers and meat meal serves for the production of protein concentrates for fattening pigs.

In this paper we describe the elaboration of methods to demonstrate the contamination of animal food with anabolic hormones. In principle, the procedure involves high-performance liquid chromatographic (HPLC) procedures followed by immunological quantitation. Medroxyprogesterone acetate (MPA), estradiol benzoate (E_2B) and NT decanoate found in illicit cocktails [3–5] can be separated by a first HPLC system with a polar bonded phase. To obtain higher selectivity, the HPLC fractions are reanalysed using a second HPLC system based on an RP-18 stationary phase. Especially the detection of E_2B promised to be one of the best markers of parenteral application, because E_2B is hydrolysed rapidly in the circulation [6]. Owing to a weak cross-reactivity of E_2B with the antibodies against estradiol, the E_2B fraction from the second HPLC treatment is hydrolysed and a third HPLC system is used to give small fractions around estradiol-17β prior to immunoassay. Further, the determination of NA and NT-17α, major metabolites of NT-17β, which are not commercially available, should give evidence of the body passage of NT esters and should therefore indicate processing of NT-treated animals. Thus an additional aim was the determination of trace amounts of NT metabolites with high reliability. We believed that this was achievable by two HPLC treatments followed by sensitive immunoassay.

EXPERIMENTAL

Origin of samples

Meat meal 1 was kindly provided by a Bavarian company. Meat meals 2–6 and crude fats a–c were obtained from about 200 veal calves per sample under supervision of veterinary officers; these animals were slaughtered because concentrations of up to 88 pg/ml NT were found in the urine. Crude fats d and e and milk replacers IV–VII were provided by a manufacturer of milk replacers. Milk replacers I–III and VIII came from veterinary officers who had confiscated them. Milk replacers IX–XI, usually fed at our experimental farm, were also analysed.

Kidney fat for control purposes was obtained from two veal calves fed MPA, E_2B and NT decanoate in a dose of 20 μg per head daily for one week followed by enlargement of the dose to 200 μg each per animal daily for two weeks [7].

High-performance liquid chromatography

Only the isocratic mode was used for elution. The times for collecting individual steroids were calibrated by UV–visible amounts or tritiated steroids prior to each assay.

The following HPLC systems were used:

System I. Eluent A, acetonitrile–20 m*M* Tris acetate buffer (pH 7.2) (42:58);

flow-rate, 1 ml/min; temperature 25°C; column 1, silica gel RP-18 Ultrasphere ODS, 5 μm (250 mm×4.6 mm) (Altex, Beckman, Munich, F.R.G.).

System II. Eluent B, methanol–20 m*M* Tris acetate buffer (pH 7.2) (65:35); flow-rate, 1 ml/min; temperature, 25°C; column 1.

System III. Eluent C, *n*-hexane–ethyl acetate (90:10); flow-rate, 2 ml/min; temperature, 30°C; column 2, LiChrospher Diol, 5 μm (50 mm×4 mm I.D.) (Merck, Darmstadt, F.R.G.).

System IV. Eluent D, methanol–20 m*M* Tris acetate buffer (pH 7.2) (70:30); flow-rate, 1 ml/min; temperature, 25°C; column 3, LiChrospher RP-18, 5 μm (50 mm×4 mm I.D.) plus (250 mm×4 mm I.D.) (Merck).

System V. Eluent E, *n*-hexane–ethyl acetate (95:5); flow-rate, 2 ml/min; temperature, 30°C; column 2.

System VI. Eluent F, methanol–20 m*M* Tris acetate buffer (pH 7.2) (90:10); flow-rate, 1 ml/min; temperature, 25°C; column 3.

Methods for residue determination

Determination of NT-17β, NT-17α and 19-norandrostenedione (NA). Samples were analysed for NT-17β, NT-17α and NA by two HPLC runs, followed by enzyme immunoassay (EIA) as described for kidney fat [2,8].

Briefly, 25 g of milk replacer or meat meal were suspended in methanol–water (20:80), extracted with light petroleum and concentrated. The remaining fat or 6 g of crude fat were dissolved in 10 ml of methanol, frozen for 1 h at −60°C and centrifuged for 3 min at −25°C. The supernatant was decanted and the residue was re-extracted with 10 ml of methanol. The combined supernatants were evaporated and the residue was dissolved in 4 ml of methanol–water (80:20), washed twice with 2 ml of light petroleum and diluted with water to <30% methanol for further purification on reversed-phase silica gel cartridges [2]. The eluate was evaporated, the residue was dissolved in 0.6 ml of acetonitrile–water (25:75) and 0.5 ml of the solution was subjected to HPLC with system I. NA and NT-17α were collected in a common fraction and NT-17β in a separate fraction (1 ml each). Both fractions were evaporated and the residue was dissolved in methanol–water (20:80) and subjected to HPLC with system II. The determination of all three 19-norsteroids in the collected fractions (0.3 ml) by EIA has been described previously [8].

Determination of medroxyprogesterone acetate (MPA). Fat samples were extracted with methanol as described above for NT metabolites. The evaporated organic extracts were dissolved in light petroleum and further steps were carried out as described recently [7].

Briefly, extracts were purified using Diol silica gel cartridges. The eluate was concentrated, dissolved in 0.6 ml of light petroleum and 0.5 ml of the solution was subjected to HPLC with system III. MPA was collected in a single fraction (1.5 ml). After evaporation of the solvent, the residue from this fraction was dissolved in 0.8 ml of methanol–water (40:60), two 0.1-ml samples were analysed by direct radioimmunoassay (RIA) and 0.5 ml was subjected to HPLC with system IV.

The fractions (0.3 ml) around MPA were evaporated and measured by RIA. All results were corrected for losses of MPA over the whole procedure by external standardization with [^{3}H]MPA.

Determination of estradiol benzoate (E_2B). Samples were extracted and purified as described above for MPA. The only difference in the extraction was that milk replacer and meat meal were suspended in methanol–water (50:50) instead of (20:80). The eluate from the Diol silica gel cartridge was dissolved in 0.6 ml of light petroleum and 0.5 ml of the solution was subjected to HPLC with system V. The E_2B fraction (1.5 ml) was collected and reanalysed by HPLC with system VI. The E_2B fraction from the second HPLC treatment was evaporated and the residue was dissolved in 0.1 ml of methanol and hydrolysed to estradiol at 25°C for 15 min with 0.1 ml of 0.5 *M* sodium hydroxide solution. After neutralization with 0.6 ml of 0.1 *M* acetic acid, two 0.1-ml samples were used for screening immunoassay and 0.5 ml was subjected to HPLC with system I. The fractions around estradiol-17β (E_2) were collected to produce parallel RIA and EIA immunograms with antisera of different specifities. The RIA was performed with antibodies against E_2 6-CMO–BSA (CMO = carboxymethyloxime; BSA = bovine serum albumin) [9] and for EIA [10] antibodies against E_2 17-HS–BSA (HS = hemisuccinate) were used. All results were corrected for losses of E_2B over the whole procedure by external standardization with [^{3}H]E_2B.

Determination of 19-nortestosterone esters. Milk replacer (4 g) or meat meal (4 g) was suspended in 4 ml of methanol–Tris acetate (pH 7.2) (50:50) and extracted twice with 10 ml of light petroleum with strong shaking for 30 min. The extract was concentrated to 4 ml and 0.2 ml was evaporated and hydrolysed with 0.5 ml of 1 *M* sodium methoxide for 15 min at 25°C. For purification the hydrolysate was diluted with 125 μl of water and washed twice with 2 ml of light petroleum. After further dilution with 2 ml of water the hydrolysate was extracted with 4 ml of *tert.*-butyl methyl ether (Merck). According to the results of a screening EIA, an aliquot of the *tert.*-butyl methyl ether extract was evaporated and the residue was dissolved in acetonitrile–20 m*M* Tris acetate buffer (pH 7.2) (25:75) and subjected to HPLC with system I, followed by EIA.

Crude fat (1 g) was dissolved in 9 ml of methanol by heating the sample to 55°C. After shaking the solution for 30 min at 25°C and centrifugation (1800 *g* for 5 min at 25°C), 1 ml of the supernatant was hydrolysed with 1 ml of 1 *M* sodium methoxide. Further steps were as for milk replacer and meat meal.

All results were corrected for procedural losses as calculated by internal standardization of all samples with testosterone cypionate. Testosterone resulting from hydrolysis was measured by RIA in the same HPLC run as used for the separation of NT.

RESULTS

No E_2B was detectable in kidney fat from untreated animals and calves fed 200 μg of E_2B per head daily for two weeks. Even seventeen days after injection of 100 mg of E_2B the residues were below 10 pg of E_2B per gram kidney fat. In contrast, kidney fat spiked with 100 pg/g E_2B gave a clear peak (Fig. 1). In veal

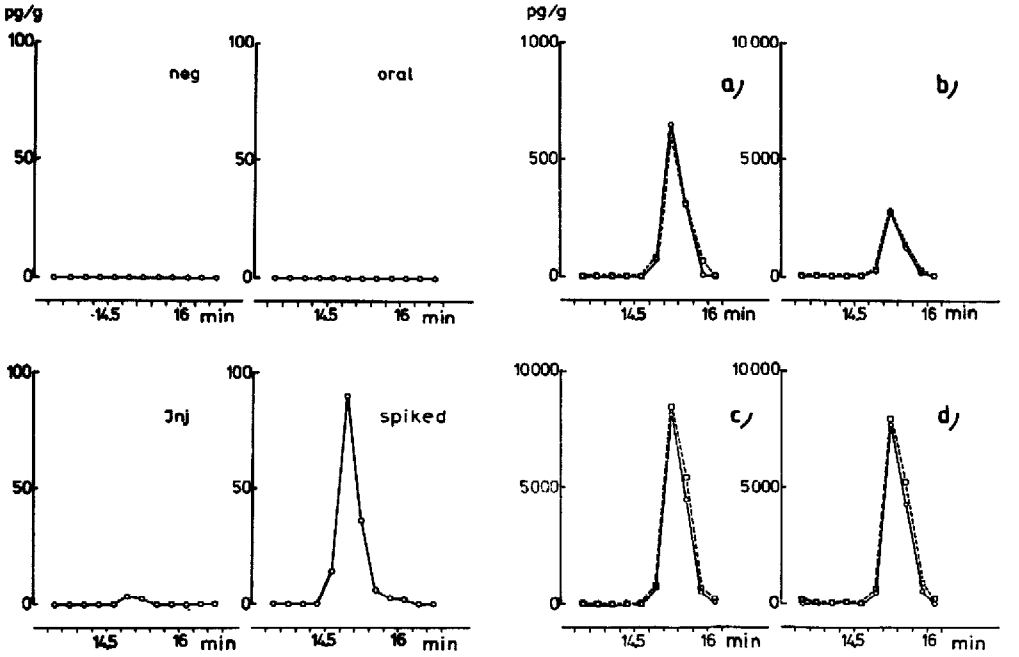

Fig. 1. Results of control sample analysis. Control samples were kidney fats from untreated calves (neg.), veal calves fed 200 μg of E_2B per head daily for two weeks and slaughtered without a waiting period (oral), after injection of 250 mg of E_2B and a waiting period of 17 days (inj.) and spiked with 100 pg of E_2B per g of fat prior to extraction. After extraction and prepurification with a Diol silica gel cartridge, E_2B was separated using two HPLC systems with different selectivity (systems V and VI). The E_2B fraction from the second HPLC run was hydrolysed and the resulting estradiol-17β was determined by HPLC (system I) followed by RIA (antibody against E_2B 6-CMO-BSA).

Fig. 2. Results of crude fat analysis. Crude fats a–c were obtained from homogenized veal calves suspected of illegal treatment. Fat d was a commercial rendered fat (of unknown origin). Samples were analysed as described in Fig. 1; in addition, an EIA was carried out with an antibody raised against E_2 17β-HS-BSA. ○, RIA; □, EIA.

calves suspected of illegal treatment, after homogenization and processing into meat meal and crude fat, E_2B was found. Although kidney fat and rendered fat are not completely comparable, confirmation of analysis by using two different antibodies showed no difference between the RIA and EIA results (Fig. 2).

The concentrations of E_2B in crude fats a–c from animals that were NT-positive after examination of urine were 21 ng/g. In meat meals 2–6 from similar NT-positive livestock herds 0.2–0.7 ng/g E_2B was measured (Table I). Only meat meal 2 and crude fat a were obtained from one group of veal calves. Between the other meat meals and fat samples there was no relationship. The concentration of E_2B in commercial crude fats d and e from a rendering plant were in the same range as obtained for crude fat from NT-positive animals. The concentrations of NT esters in meat meals and crude fats were considerably higher, without exception. NT esters were calculated as NT decanoate, because in pilot experiments with a method analogous to the determination of E_2B NT decanoate had been

TABLE I

ANABOLICS IN MEAT MEAL AND CRUDE FAT (ng/g)

Sample	NT-17β	NA	NT-17α	MPA	E_2 benzoate	NT ester[a]
Meat meal:						
1	—[b]	—	—	<0.01	<0.04	<0.07
2	—	—	—	<0.01	0.6	202
3	—	—	—	<0.01	0.2–0.7	48–161
Crude fat from animals suspected of illegal treatment:						
a	—	—	—	—	1.5	267
b	3.8	0.2	0.1	0.10	6.3	>300
c	4.1	0.3	0.1	0.20	21	>300
Commercial crude fat from a rendering plant:						
d	3.0	0.6	0.3	31	21	117
e	—	—	—	8	11	40

[a]Calculated as NT decanoate.
[b]Hormones not analysed.

TABLE II

ANABOLICS IN MILK REPLACERS (ng/g)

Milk replacer	NT-17β	NA	NT-17α	MPA	E_2 3-benzoate	NT ester[a]
I–III	—[b]	—	—	—	—	<0.46
IV	0.6	0.2	0.02	9.0	1.6	36
V	0.3	0.1	0.02	6.5	0.6	22
VI	0.8	0.15	0.02	11.5	1.3	57
VII	0.6	0.1	0.04	7.7	3.5	57
VIII–XI	<0.01	<0.01	<0.01	<0.01	<0.01	<0.75

[a]Calculated as NT decanoate.
[b]Hormones not analysed.

identified in crude fats b and d. However, MPA was not detectable in meat meals and the amounts in crude fats b and c from animals suspected of illegal treatment were low. In comparison, the concentration of MPA was relatively high in commercial crude fats d and e. NT-17β, NA and NT-17α were found in all crude fat samples investigated (b–d) and the ratios of the concentrations were about the same for both types of crude fat (Table I).

In milk replacers I–III and VIII–XI, residues of the anabolics mentioned above were not detectable. However, all steroid esters and NT metabolites investigated could be found in milk replacers IV–VII sampled in the same period as crude fat d (Table II) and the relative concentrations of the single residues were similar.

DISCUSSION

Methods

The separation of HPLC systems I and II [2] and the validity of the immunoassays for MPA [7] and the NT metabolites [2,8] had been described previ-

ously. The results presented here show that E_2B and NT esters can also be measured even at very low levels in the picogram range. E_2B is purified by normal-phase chromatography and separated from other estradiol esters by reversed-phase chromatography prior to hydrolysis and final determination. Total NT esters are first hydrolysed and the resulting NT is purified by HPLC before immunoassay. Finally, no signal was observed in the immunograms of negative samples, which demonstrates that all interfering substances from the investigated materials were removed and proves the reliability of the methods.

Importance of residues

E_2B was not detectable in kidney fat from veal calves fed E_2B. Even kidney fat from a calf that contained an injection site with 1.9 mg of E_2B on the day of slaughter showed less than 10 pg/g E_2B, which can be explained by the rapid hydrolysis of E_2B in blood. Therefore, the detection of E_2B in animals or animal products is a marker of parenteral application of this anabolic compound. The simultaneous presence of NT esters in animals supports the proof of illegal application. With regard to these results, the detection of E_2B and NT esters in commercial crude fat and milk replacers gives evidence of processing of animals with injection sites. On the other hand, intentional addition of anabolics to milk replacers or crude fat used for the production of milk replacers seems improbable, because NA and NT-17α were found, which are not available commercially. Further, the pattern of anabolic residues in commercial crude fat and milk replacers coincides with that of crude fat from animals suspected of illegal treatment. Differences in the concentrations of E_2B and NT esters between the crude fat samples a–c are caused by various waiting periods until slaughter. The concentrations of MPA in commercial crude fats d and e are much higher than those in fats a–c. This may be due to the application of illegal cocktails with higher MPA contents or to other sources of contamination, such as processing of bitches and cats legally treated with MPA preparations to prevent heat.

An exact comparison of anabolic residues in fat and milk replacers is difficult because the analysis of only one commercial crude fat (d) from one production run is not representative. Nevertheless, the residues of all the anabolics, especially the high values of MPA in milk replacers IV–VII, are in good agreement with those of fat d. The concentrations of E_2B and NT esters in rendered fat resulting from the application of registered veterinary preparations should be strongly reduced by commercial fat from untreated animals. Further, only up to 50% of the total fat processed into milk replacers is animal fat. Therefore, the detection of E_2B and NT esters in some milk replacers suggest a strong abuse of anabolics in animal production in certain regions. Hence the uncontrolled, widespread illegal use of anabolic hormones in the EEC raised a new forensic problem, namely that xenobiotic hormones monitored in urine samples do not give evidence of the illegal treatment of the animals.

Normal feeding of milk replacer IV resulted in up to 2 ng/ml NT in the urine [1]. After the determination of NT ester residues in this milk replacer we calculated a feeding dose of 37 μg of NT ester per animal daily. For comparison, the excretion of NT in urine was measured using two veal calves that were fed for

TABLE III

NT CONCENTRATIONS IN URINE AFTER ORAL AND PARENTERAL APPLICATION OF NT ESTERS

Sample	NT in urine (ng/ml)
Milk replacer IV	
37 μg of NT ester per head daily	-2
Feeding trial:	
20 μg of NT decanoate per head daily	0.2 -2.1
200 μg of NT decanoate per head daily	3.5 -9.5
Registered NT ester preparation:	
Single i.m. or i.v. injection of 100 mg of NT ester:	
days 1-7	50-10
after day 7	< 10
Illegal 'cocktail' with NT decanoate:	
Single i.m. injection of 200 mg of NT decanoate:	
days 1-17	115-10
Three i.m. injections of 200 mg of NT decanoate:	
days 1-29 after last injection	125-10

one week 20 μg of NT decanoate, MPA and E_2B per head daily followed by an increase in the dose to 200 μg per animal daily for two weeks [11]. With respect to the usual fluctuations in excretion and composition of urine, the concentration of NT ester found in milk replacer IV and the amount of NT decanoate orally applied agree very well with the residues of NT in urine (Table III). Concentrations of more than 10 ng/ml NT in urine may be possible if 200 μg or more of NT ester per head daily are fed (about 200 ng of NT ester per gram of food). This is three to four times more than that found in contaminated milk replacers. Concentrations higher than 10 ng/ml NT in urine were observed after injection of calves with registered NT ester preparations [12] and illicit cocktails. Variations in the amounts and duration of NT residues in urine are due to different doses; the half-life for excretion of NT in urine was the same for registered and illicit preparations [11].

Finally, although contamination of milk replacers has caused forensic problems, the elucidation of the comtamination chain and the development of analytical methods now offer several ways of differentiating between oral and parenteral application of anabolics.

REFERENCES

1 E. Rattenberger and P. Matzke, Arch. Lebensmittelhyg., 39 (1988) 21.
2 H.H.D. Meyer, F.-X. Hartmann and M. Rapp, J. Chromatogr., 489 (1989) 173.
3 E.H.J.M. Jansen, H. van Blitterswijk and R.W. Stephany, Vet. Q., 6 (1984) 60.
4 H. Karg and H.H.D. Meyer, VDLUFA-Schriftenreihe, 20 (Kongressband 1986) 29.
5 M. Rapp and H.H.D. Meyer, Arch. Lebensmittelhyg., 38 (1987) 35.
6 T.G. Dunn, C.C. Kaltenbach, D.R. Koritnik, D.L. Turner and G.D. Niswender, J. Anim. Sci., 46 (1977) 659.
7 M. Rapp and H.H.D. Meyer, Food Addit. Contam., 6 (1989) 59.

8 H.H.D. Meyer and P. Funcke, in S. Görög (Editor), Proceedings of the 3rd Symposium on the Analysis of Steroids, Sopron, 20–22 October 1987, Akadémiai Kiadó, Budapest, 1988, p. 225.
9 H.H.D. Meyer, M. Landwehr, D. Schopper and H. Karg, Food Addit. Contam., 1 (1984) 261.
10 E. Möstl, H.H.D. Meyer, E. Bamberg and E. Hegel, in S. Görög (Editor), Proceedings of the 3rd Symposium on the Analysis of Steroids, Sopron, 20–22 October 1987, Akadémiai Kiadó, Budapest, 1988, p. 219.
11 M. Rapp, P. Funcke and H.H.D. Meyer, Arch. Lebensmittelhyg., 39 (1989) 114.
12 E. Rattenberger, P. Matzke, A.P. Funke and H.H.D. Meyer, Arch. Lebensmittelhyg., 38 (1987) 73.