Nachweis von Rückständen anaboler Wirkstoffe in Fleisch und Harn von Masttieren.

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Ein multi-residue Nachweisverfahren wird beschrieben in dem anabole Steroide im Bereich von 0.5 ppb im Fleisch und Harn erfaszt werden.
Nach enzymatischer Hydrolyse von Muskelhomogenaten (50 g) werden die Hormone mit 80 %-igem Methanol extrahiert. Nach Entfetten werden die Hormone extrahiert mit Dichlormethan. Zur weiteren Reinigung des Extraktes werden die Steroïde auf einer Amberlite XAD-2 Saüle adsorbiert und eluiert mit Methanol. Mit Hilfe einer gekoppelter Celite-KOH und Al_2O_3-Saüle werden die Hormone getrennt in eine Östrogen- und nicht-Östrogen Fraktion. Harn wird analog behandelt : nach Adsorption der Hormone an einer XAD-2 Saüle wird das Eluat enzymatisch hydrolysiert.
Nach bidimensionaler Dünnschichtchromatographie (HPTLC) der Extrakte werden die Hormone lokalisiert an Hand der Schwefelsaüre induzierten Fluoreszenz unter UV-licht. Die spezifische Fluoreszenzfarbe sowie die stabile Rf-Werte ermöglichen einen genauen Nachweis im Nanogram-Bereich von Rückständen verschiedener anabolen Wirkstoffe in Extrakten von Muskelfleisch und Harn. Durch Messung der Fluoreszenzintensität der Flecke werden die Hormone quantitativ erfaszt bei einem Variationskoeffizient von 10 %.

Detection of steroïd residues in meat and urine of slaughter animals.

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A multiresidue method has been developed allowing routine detection of anabolic steroids in meat and urine contaminated at levels as low as 0.5 ppb (10 parts per 10^9).
Following enzymic hydrolysis of muscle tissue (50 g), all hormones are extracted in 80 % methanol. The filtrate is defatted and the steroïds extracted in dichloromethane. Clean-up of the extracts is performed by adsorption of the hormones on an Amberlite XAD-2 column and elution with methanol. By passage through a coupled celite-KOH and Al_2O_3-column the hormones are separated into an estrogen- and non-estrogenfraction. A similar extraction scheme for urine samples starts with the adsorption of hormones on Amberlite XAD-2 column followed by enzymic hydrolysis.
After bidimensional thin-layer chromatography (HPTLC) of the extracts, the hormones are evaluated under U.V.-light after sulfuric acid induced fluorescence. The fluorescence colour and relative mobility of the steroïds permit reliable detection of nanogram amounts of different anabolic substances in meat or urine extracts. Through scanning of the fluorescence intensity on the plates, the amount of hormones may be determined with an accuracy of $\pm$ 10 %.

L 11:2

Détection des résidus d'hormones stéroïdes dans les viandes et l'urine des animaux de boucherie.

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Une méthode fût développée permettant le dépistage simultané de plusieurs résidus d'anabolisants dans la viande ou dans l'urine au niveau de 0.5 ppb.
Après hydrolyse enzymatique du tissu musculaire (50 g) les hormones sont extraites par 80 % méthanol. Après délipidation, les stéroïdes sont extraits de la solution méthanolique par du dichlorométhane. Les extraits sont purifiés par adsorption des stéroides sur une colonne Amberlite XAD-2 et élution par le méthanol. En passant l'extrait par une colonne celite-KOH couplée à une colonne d'Al_2O_3 les hormones sont séparées dans une fraction oestrogène et une fraction non-oestrogène. Un schéma similaire a été élaboré pour l'urine : les hormones sont d'abord adsorbées sur une colonne Amberlite XAD-2 suivis par hydrolyse enzymatique.
Après chromatographie des extraits sur couche mince (HPTLC) les hormones sont révélées par acide sulfurique et localisées par la fluorescence produite par excitation sous lumière U.V. La couleur de la fluorescence ainsi que la mobilité relative des stéroïdes permet la détection simultanée du plusieurs substances anabolisants dans les extraits de la viande ou de l'urine. En mesurant l'intensité de la fluorescence des taches sur couche mince, la quantité des hormones fût déterminée avec une réproducibilité de $\pm$ 10 %.

Обнаружение остатков стероидных гормонов в мясе и моче убойного скота

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Разработан метод, позволяющий обнаружить одновременно несколько видов анаболических остатков в мясе или в моче на уровне 0,5 ч.н.б.
После проведения ферментативного гидролиза мышечной ткани /50 г/, гормоны экстрагируются с помощью 80 %-ного раствора метанола. После обезжиривания стероиды извлекаются из метанолового раствора посредством дихлорметана. Экстракты очищаются путем адсорбции стероидов на колонке Amberlite XAD-2 и элюирования метанолом. При прохождении экстракта через целитовую колонку KOH, соединенную с колонкой, содержащей Al_2O_3, гормоны разделяются на эстрогенную и неэстрогенную фракции. Аналогичная схема разработана и для анализа мочи: сперва адсорбируют гормоны на колонке Amberlite XAD-2, а затем проводят ферментативный гидролиз.
После хроматографии экстрактов на тонком слое (HPTLC), гормоны извлекаются с помощью серной кислоты и количество их определяется по флуоресценции, возникающей в результате ультрафиолетового облучения. Цвет флуоресценции и относительная подвижность стероидов позволяют одновременно обнаружить в экстрактах мяса или моче несколько анаболических веществ. Путем измерения интенсивности флуоресценции на тонком слое определяют количество гормонов с точностью до $\pm$ 10 %.

Detection of steroid residues in meat and urine of slaughter animals

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Introduction

The use of anabolic steroids as growth promoting agents has become a wide-spread practice though regulations in most EEC-countries prohibit the use of these substances in cattle breeding. The variety of combinations used and the lack of sensitive, reliable multiresidue screening procedures hampered an efficient control on the abuse of these substances[1]. A convenient, technically simple procedure is presented, permitting routine detection of most steroid residues in meat and urine down to a 0.5 ppb-level. This objective is realised through development of a suitable clean-up procedure from meat and urine, analysis of the extracts by bidimensional chromatography on nanogram plates and evaluation of the hormones by sulfuric acid induced fluorescence.

Material and Methods

The following apparatus were used : homogeniser (Ultra-Turrax), vacuum evaporator, Blackray transilluminator (Ultra-Violet Products, Inc. U.S.A.), Zeiss KM3 chromatogram spectrophotometer for quantitative U.V.-reflectance or fluorescence measurements and a Tri-Carb liquid scintillation counter (Packard, U.S.A.).

Most reference steroids were purchased from steraloids (Wilton, U.S.A.). Radioactive steroids (^{3}H-diethylstilboestrol, ^{3}H-estradiol, 4-^{14}C testosterone, 4-^{14}C progesterone) were purchased from Amersham (Bucks, G.B.). ^{3}H-trenbolone was a gift from Roussel Uclaf (France). Celite 545 (20 - 45 µ) and Amberlite XAD-2 (300 - 1000 µ) were obtained from Serva and Al_2O_3 (neutral, activity Brockman I) was a product from Woelm (G.F.R.). Glucuronidase-sulfatase (Succus Helix Pommatia) was purchased from I.B.F. (France) and diethylether, free from peroxides, from Gifrer & Barbezat, Décines, France. All other reagents were analytical grade from E. Merck (Darmstadt). Silicagel 60 nanogram plates were supplied by E. Merck.

Pre-treatment of Amberlite XAD-2 : 50 g of resin is suspended in 250 ml of methanol in a graduated cylinder. After 2 min. sedimentation, the supernatant is siphoned and the procedure repeated twice. The resin is washed on a Buchner funnel with several volumes distilled water and stored at 4 °C.

Extraction of hormones from meat :

Minced meat (50 g) is homogenised in presence of 50 ml sodium acetate buffer (0.04 M,pH 5.2). After addition of 0.5 ml glucuronidase-sulfatase the hormones are hydrolysed overnight at 37 °C. The incubation mixture is homogenised in presence of 180 ml methanol and centrifuged (10 000 g, 10 min.). Fat is removed by extracting the supernatant two times with 50 ml n. hexane. The methanol phase is extracted with 150 ml dichloromethane and the lower phase collected. The upper phase is then successively reextracted with 90 ml and 80 ml dichloromethane. The combined dichloromethane extracts are evaporated, dissolved in 20 ml distilled water and passed through an Amberlite XAD-2 column (80 x 10 mm). The column is washed with 40 ml distilled water. The steroids are eluted by passing 40 ml of methanol through the column. The methanol eluate is evaporated to dryness at 40 °C.

L 11:4

Extraction of hormones from urine :

Urine (50 ml) is percolated through an Amberlite column (80 x 20 mm). After washing the column with 100 ml distilled water, the conjugated and unconjugated steroids are eluted with 100 ml of methanol. The eluate is concentrated to a volume of ca. 1 ml on a rotary evaporator at 40 °C. The residue is taken up in 10 ml acetate buffer (0.2 M, pH 5.2). The enzymdigest is diluted with 20 ml methanol and extracted with 15 ml dichloromethane. The lower phase is collected and the upper phase reextracted with 2 times 10 ml dichloromethane. The pooled dichloromethane phases are evaporated to dryness at 40 °C.

Clean-up of the extracts :

Clean-up and separation of the hormones in an estrogen and non-estrogen fraction is performed in one step by passage of the extracts through a celite-KOH column coupled to an Al_2O_3-column.
A celite column is prepared by pouring an intimate mixture of 2 g celite with 1 ml 0.3 N KOH, slurried in 10 ml benzene, into a chromatographic column (200 x 9 mm). The celite is gently tamped with a glass rod to a height of ca. 6 cm. Successively 15 ml water-washed diethylether, 10 ml benzene and 15 ml benzene-isooctane (50:50, v/v) are passed through the column.
The Al_2O_3-column is prepared in pouring a benzene suspension of 4 g neutral Al_2O_3 into a 200 x 9 mm column. After draining the benzene, the column is prewashed with 15 ml benzene-isooctane (50:50, v/v).
The celite column is mounted on top of the Al_2O_3 column. Meat or urine extracts are dissolved in 5 ml benzene-isooctane (50:50, v/v), quantitatively transferred on top of the celite column and allowed to drain into the column. Rinse evaporation tube twice with 5 ml benzene-isooctane and add rinses on top of celite column. After draining the solutions into the celite surface, the columns are uncoupled.
The celite column is first washed with 50 ml benzene-isooctane (50:50, v/v) after which the estrogens are eluted with 50 ml water-washed diethylether. The eluate is evaporated to dryness.
The Al_2O_3-column is washed with 25 ml benzene-isooctane (50:50, v/v). The androgens and progestagens are quantitatively eluted from the Al_2O_3-column with 30 ml benzene-ethanol (99:1, v/v). This eluate is evaporated to dryness. The concentrated extracts are dissolved in 3 x 1 ml acetone, quantitatively transferred into a conical tube and concentrated to 50 µl under a stream of nitrogen.

Thin-layer chromatography :

The extracts are analysed by bidimensional TLC using silicagel 60 nanogram plates. Development is carried out in non-saturated tanks. Maximum 5 µl of the extract is spotted on a nanogram plate. In the side lanes appropriate concentrations of the reference mixtures (containing 10 - 80 ng of each steroid) are applied. The chromatographic development of the plate is carried out over a distance of 7 cm using the appropriate solvent systems (Tables 1 and 2). The plates are air-dried and the fluorescence reaction is induced by spraying estrogens with 5 % sulfuric acid in acetic acid anhydride or by spraying androgens with 5 % sulfuric acid in ethanol. The plate is dried for 15 min. at 100 °C and the fluorescence viewed by transillumination at 366 nm.
Experimental parameters for fluorescence measurement were M-Pr setting on the Zeiss instrument operated with a mercury lamp, with excitation at 366 nm and emission using a cut-off filter FL 46.

Results

Chromatography and fluorescence detection :

Several solvent systems were tried using standard solutions. Solvent combinations, indicated in tables 1 and 2, gave more concentrated spots, showed good separation among the different anabolics and presented a dark background when transilluminated under U.V.-light. Moreover the solvent systems produced a sharp differentiation of the hormones tested with spots originating from substances present in meat or urine extracts. This fact allowed bidimensional TLC analysis of hormones after spotting 1/5 of the total extract on the plate.
The sulfuric acid test is known to be highly selective in producing characteristic colour and fluorescence responses in respect to minor structural alterations in steroids[2]. This sensitive test was optimalised in studying the response of various sulfuric acid sprays on different hormones in combination with different heating times and oven temperatures. With the exception of trenbolone(acetate), where 5 min. heating time proved optimal, most steroids showed maximal response and fluorescence stability after 15 min. incubation at 100 °C. As little of 0.5 - 3 ng of most steroids may be detected (Tables 1 and 2). However, detection of hormones in meat or urine extracts not only depends from its detection limit : spot broadening during development and contrast of the spots with the background are even more important. As seen from tables 1 and 2, a low detection limit was observed after adding different free steroids to meat. Urine extracts produce a higher background : the detection limit was approximately twice that observed with meat extracts.
Optimal activation of most steroid derivatives was observed at 366 nm with fluorescence maxima between 490 and 550 nm. Steroids showed a linear relation between concentration and fluorescence (as area of recorded signal) in the range of 2 times up to 10 times the detection limit. Since the fluorescence measured depends on the solvents used during development, quantitative determination of the hormones following bidimensional chromatography involved an adaptation of the antidiagonal technique described by Beljaars et al[3]. Non-fluorescent derivatives (e.g. dienestrol) involves U.V. measurement in the transmission-reflectance mode. For diethylstilboestrol (DES) this technique was 10 times more sensitive than the induced fluorescence described by Schuller[4]. The standard deviation of the individual values of the different hormones ranged between 2.5 - 6 % in all instances if scanning was performed within 4 hrs after development.

Analyses of extracts from muscle tissue and urine :

The overall recovery of radioactive hormones, added to 50 g muscle tissue or urine was determined. ^{14}C-testosterone or ^{3}H-trenbolone were recovered in 82.6 % yield : reproducibility was 5.2 % (n = 12). Estrogens (^{3}H-DES, ^{3}H-estradiol) added to meat were recovered in 72 ± 6.5 % yield (n = 5). However, the mean recovery of estrogens from urine was only 51.0 ± 5.5 % (n = 11). Hormones added to meat or urine samples were quantitatively determined using the scanning technique : yields were similar to that found with the radioactive hormones with a reproducibility of ± 10 % (n = 20).
Monoglucuronides of DES, dienestrol and hexoestrol, added to urines in concentrations of 2 - 100 ppb, were recovered in yields of 26 ± 3.8 % (n = 9). Monoglucuronide hydrolysis was found to be inhibited for 50 % by different urine extracts in contrast to hydrolysis performed in water or meat extracts.
Meat extracts from male calves, containing 1 - 5 ppb methyltestosterone as found by TLC analysis, were analysed by GLC-mass spectrometry in the laboratory of Ribermag (Reuil Malmaison, France). The concentrations obtained by mass-fragmentometry were similar with those from TLC : the coefficient of variation between the two methods was ± 15 % (n = 4). Direct injection of an aliquot of the extract into the GLC-mass spectrometer allowed determination of 0.05 ppb methyltestosterone in meat samples.

Table 1.
Rf-values and detection limit of some estrogens on nanogram plates by sulfuric acid induced fluorescence.
Solvent systems : 1 = n.hexane-diethylether-dichloromethane (4:3:2) ; 2 = chloroform-ethanol-benzene (90:2.5:10).

Substance	Solvent system		Fluorescence	Detection limit	
	1	2	(366 nm)	plate (ng)	meat (ppb)
17β-estradiol	0.39	0.22	yellow	3	1
17α-estradiol	0.34	0.23	yellow	2	0.5
estriol	0.01	0.03	yellow	10	2
estrone	0.58	0.37	yellow	10	2
ethynylestradiol	0.50	0.29	brown-yellow	12	4
DES trans	0.59	0.21	red colour	2	0.5
cis	0.33	0.19	red colour	2	0.5
dienestrol	0.53	0.17	red colour	2	0.5
hexoestrol	0.56	0.20	green	10	2
zeranol	0.17	0.18	green colour	40	10

Table 2.
Rf-values and detection limit of some androgens and progestagens on nanogram plates by sulfuric acid induced fluorescence.
Solvent systems : 2 = chloroform-ethanol-benzene (90:2.5:10) ; 3 = n.hexane-dichloromethane-ethylacetate (20:40:40).

Substance	Solvent system		Fluorescence	Detection limit	
	2	3	(366 nm)	plate (ng)	meat (ppb)
testosterone	0.33	0.44	yellow	2	1
methyltestosterone	0.37	0.49	green-yellow	2	0.5
19-nortestosterone	0.33	0.38	yellow	2	1
trenbolone	0.32	0.39	bright blue	1	0.2
trenbolone acetate	0.68	0.77	bright blue	3	0.2
progesterone	0.59	0.55	blue	20	5
medroxyprogesterone	0.37	0.63	yellow	2	1
chloormadinonacetate	0.61	0.74	orange	4	3
19-norethisterone	0.39	0.64	brown colour	20	5
ethynyltestosterone	0.45	0.71	yellow	20	4

Conclusions

The method described allows routine detection of different anabolic residues in meat and urine at levels of 0.5 - 5 ppb. One analyst can carry out approximately 10 analyses per week. The major advantage of this method over RIA-assays[5] and other TLC-methods[1,6,7] resides in its general applicability to detect several anabolics with a high sensitivity and reproducibility. Notwithstanding the fairly high detection level of anabolics in meat, several meat samples have been found to be contaminated at residue levels of 5 ppb with methyltestosterone, dienestrol, DES e.a. Preliminary experiments indicate that, in using the described extraction method, 0.05 ppb of several anabolics may be routinely detected in meat extracts by GLC-mass spectrometry.

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References

1. J.J. RYAN, J. Chromatogr. 127 (1976) 53 - 89.
2. E. HEFTMANN in Chromatography of Steroids, Elsevier Publ. Co, Amsterdam, 1976, p. 18.
3. P.R. BELJAARS, C.A.H. VERHÜSTDONK, W.E. PAULSCH and D.H. LIEM, J. Ass. Offic. Anal. Chem., 56 (1973) 1444.
4. P.L. SCHULLER, J. Chromatog. 31 (1967) 237.
5. B. HOFFMANN, H. KARG, K.H. HEINRITZI, H. BEHR, E. RATTENBERGER, Mitt. Geb. Lebensmittelhyg. 66 (1975) 20.
6. K. VOGT, Arch. Lebensmittelhyg. 28 (1977) 161.
7. K. OEHRLE, K. VOGT, Acta Endocrin. 82, suppl. 202 (1976) 61.