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SENSITIVE MULTI-RESIDUE METHOD FOR DETECTION OF ANABOLICS IN URINE AND IN TISSUES OF SLAUGHTERED ANIMALS

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SUMMARY

A routine procedure is described for the dependable detection of various anabolic residues in tissues or urine contaminated at levels as low as 0.5–10 ppb (10 parts per 10^9). A suitable extraction and clean-up procedure was developed, permitting adequate recovery (60–80%) of various anabolics from tissue samples (50 g) or urine (50 ml). Following two-dimensional thin-layer chromatography, the presence of the anabolic residues are detected by sulphuric acid–induced fluorescence. The detection limit of most anabolics is of the order of 1–10 ng.

INTRODUCTION

Anabolic steroids are widely used as growth-promoting agents, although regulations in most EEC countries prohibit the use of these compounds in cattle breeding[1]. The variety of anabolic combinations used illegally, the low residue levels in the tissues of the treated animals[2] and the lack of suitable multi-residue methods hamper the efficient control of the use of anabolics[3,4].

Published chemical methods are primarily concerned with the analysis of specific synthetic anabolics, mainly estrogens[3]. None of these approaches is applicable with adequate sensitivity to a variety of tissues. Moreover, most of these procedures lack specificity, which results in high background interferences giving rise to false positive diagnoses.

In the regulatory control of the abuse of hormones, the identity of the anabolic is not known, so a multiple-residue procedure must be employed. Suitable routine methods for the simultaneous detection of hormones in meat and urine should allow dependable detection of 0.5–10 ppb anabolics by a simple procedure within a limited time interval. Moreover, concordant results should be reached in different laboratories when using the method on the same samples[4].

In this paper, a convenient, specific and reliable multi-residue procedure is presented that permits the routine detection of anabolic residues in meat or urine at levels of 0.5–5 ppb.

EXPERIMENTAL

Apparatus

The following apparatus was used: homogenizer (Ultra-turrax), centrifuge, rotary vacuum evaporator with vacuum source, water-bath, extraction flasks (500 ml), chromatographic tanks, heating cabinet, UV (366 nm) transilluminator (C-62; U.V. Products Inc., San Gabriel, Calif., U.S.A.) and sample applicator.

Trivial names of anabolics

Benzestrol = 4,4'-(1,2-diethyl-3-methyl-1,3-propanediyl)bisphenol; chlormadinone = 6-chloro-17-hydroxy-4,6-pregnadiene-3,20-dione; 1-dehydrotestosterone = 17β-hydroxy-1,4-androstadien-3-one; 6-dehydrotestosterone = 17β-hydroxy-4,6-androstadien-3-one; 6-dehydroprogesterone = 4,6-pregnadiene-3,20-dione; diethylstilboestrol = DES = 4,4'-(1,2-diethyl-1,2-ethenediyl)bisphenol; dienestrol = DIEN = 4,4'-(1,2-diethylidene-1,2-ethanediyl)bisphenol; 5-dihydrotestosterone = 17β-hydroxy-5α-androstan-3-one; ethisterone = 17α-ethynyl-17β-hydroxy-4-androsten-3-one; 17α-estradiol = 1,3,5(10)-estratiene-3,17α-diol; 17β-estradiol = 1,3,5(10)-estratriene-3,17β-diol; estriol = 1,3,5(10)-estratriene-3,16α,17β-triol; estrone = 3-hydroxy-1,3,5(10)-estratrien-17-one; ethinylestradiol = 17α-ethynyl-1,3,5(10)-estratriene-3,17β-diol; equilenin = 3-hydroxy-1,3,5(10),6,8-estrapentaen-17-one; fluoxymesterone = 9-fluoro-11β,17β-dihydroxy-17α-methyl-4-androsten-3-one; hexestrol = HEX = 4,4'-(1,2-diethyl-1,2-ethanediyl)bisphenol; lynestrenol = 17α-ethinyl-17β-hydroxy-4-estrene; medroxyprogesterone = 17-hydroxy-6α-methyl-4-pregnene-3,20-dione; megestrol = 17α-hydroxy-6-methyl-4,6-pregnadiene-3,20-dione; melengestrol = 17α-hydroxy-6-methyl-16-methylene-4,6-pregnadiene-3,20-dione; methandriol = 17α-methyl-5-androstene-3β,17β-diol; mestranol = 17α-ethynyl-3-methoxy-17β-hydroxy-1,3,5(10)-estratriene; 16α-methylprogesterone = 16α-methyl-4-pregnene-3,20-dione; 16β-methylprogesterone = 16β-methyl-4-pregnene-3,20-dione; 17α-methyltestosterone = 17α-methyl-17β-hydroxy-4-androsten-3-one; 4,9,11-methyltestosterone = 17α-methyl-17β-hydroxy-4,9(11)-androstadien-3-one; norethinodrel = 17α-ethynyl-17-hydroxy-5(10)-estren-3-one; 19-norethisterone = 19-nor-17α-ethynyl-17β-hydroxy-4-androsten-3-one; 19-nortestosterone = 17β-hydroxy-4-estren-3-one; progesterone = 4-pregnene-3,20-dione; Δ^1-progesterone = 1,4-pregnadiene-3,20-dione; testosterone = 17β-hydroxy-4-androsten-3-one; trenbolone = 17β-hydroxy-4,9,11-estratrien-3-one; vinyltestosterone = 17α-vinyl-17β-hydroxy-4-androsten-3-one; zearalenone = 6-(10-hydroxy-6-oxo-*trans*-1-undecyl)-β-resorcyclic acid μ-lactone; zeranol = 6-(6,10-dihydroxyundecyl)-β-resorcyclic acid μ-lactone.

Reagents and reference compounds

Silica gel 60 thin-layer plates or micro-plates without fluorescence indicator were obtained from E. Merck (Darmstadt, G.F.R.) (Cat. Nos. 5721 and 5631, respectively), Amberlite XAD-2 (300–1000 μm) and Celite 545 (20–45 μm) from Serva (Heidelberg, G.F.R.) and neutral Al_2O_3 (Brockmann activity I) from Woelm (Eschwege, G.F.R.).

Glucuronidase–sulphatase enzyme suspension (Succus *Helix pommatia*: 10,000 Fishman units glucuronidase + 80,000 Roy units sulphatase) was obtained from I.B.F. (Clichy, France).

Most reference steroids were obtained from Steraloids (Wilton, N.Y., U.S.A.). Zeranol and zearalenone were a gift from Dr. Stephany (RIV, Bilthoven, The Netherlands). Medroxyprogesterone (acetate) was a gift from Upjohn (Kalamazoo, Mich., U.S.A.). Trenbolone, trenbolone acetate, [6,7-^{3}H]trenbolone and [6,7-^{3}H]-trenbolone acetate were gifts from Roussel-Uclaf (Paris, France). Other radioactive steroids, [monoethyl-^{3}H]diethylstilboestrol, [2,4,6,7-^{3}H]- or [4-^{14}C]estradiol, [4-^{14}C]-testosterone and [4-^{14}C]progesterone, were purchased from Amersham (Bucks, Great Britain).

Methylcellosolve (ethylene glycol monoethyl ether), sequanal grade, was purchased from Pierce (Rockford, Ill., U.S.A.), PPO (2,5-diphenyloxazole) and dimethyl-POPOP [1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene] from Packard (La Grange, Ill., U.S.A.) and diethyl ether, free from peroxides, from Gifrer & Barbezat (Decines, France).

All other reagents were reagent-grade products from E. Merck.

Pretreatment of Amberlite XAD-2[5]. Low-molecular-weight materials and fines were removed by allowing the resin to sediment in methanol. Amberlite XAD-2 (50 g) was stirred into 250 ml methanol during 3 min. After sedimentation in a graduated cylinder (500 ml; 2 min), the supernatant was siphoned off. This process was repeated three times. The resin was suspended in 250 ml distilled water and decanted after 5-min sedimentation. The resin was stored in the moist form.

Pretreatment of Al_2O_3. In a glass-stoppered flask, 20 g Al_2O_3 (Brockmann activity I) were treated with 1.2 ml distilled water. After vigorous agitation, the Al_2O_3 was stored overnight at room temperature.

Solutions

Stock solutions of the hormones in methanol were prepared at a concentration of 100 μg/ml.

Chromatographic solvent systems: 1 = *n*-hexane–diethyl ether–dichloromethane (4:3:2); 2 = chloroform–ethanol–benzene (36:1:4); 3 = *n*-hexane–dichloromethane–ethyl acetate (2:4:4).

Columns

XAD-2 column. (I) Approximately 8 g resin were slurried in 30 ml distilled water and packed into a column (200 × 10 mm). The resin was washed with 50 ml distilled water. (II) Alternatively, 35 g of resin was suspended in three volumes of distilled water and packed into a column (300 × 20 mm). The resin was washed with 100 ml distilled water.

Celite column. An intimate mixture of 2 g Celite with 1 ml 0.3 *N* KOH, slurried in 10 ml of benzene, was poured into a column (200 × 9 mm) provided with a PTFE stopcock and a male glass-joint. The Celite was gently tapped with a glass rod to give a height of 6 cm. The column was successively washed with 15 ml water-washed diethyl ether, 10 ml benzene and 15 ml benzene–isooctane (1:1).

Al_2O_3 column. A suspension of 4 g Al_2O_3 in 10 ml benzene was poured into a (200 × 9 mm) column provided with a PTFE stopcock each and ending with a female glass-joint. After draining the benzene, the column was washed with 15 ml benzene–isooctane (1:1).

Extraction of hormones from tissue

The main steps in the procedure during analysis of anabolics from animal tissues are shown in Fig. 1.

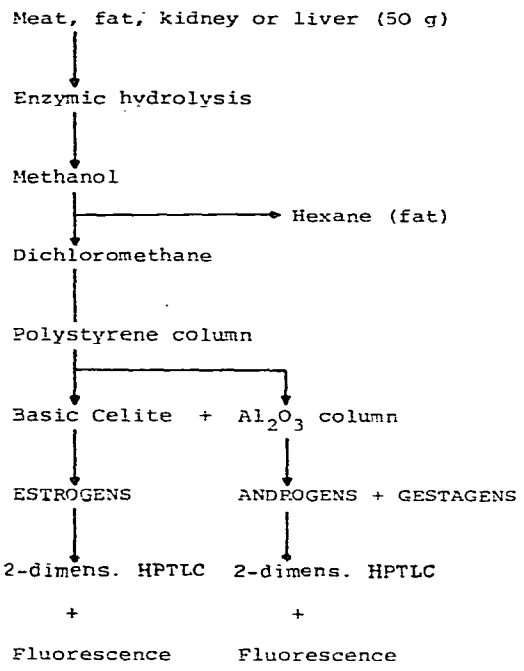

Fig. 1. Scheme for detection of anabolic steroids in animal tissues.

Approximately 50 g of minced tissue (meat, liver, kidney) were homogenized in the presence of 50 ml sodium acetate buffer (0.04 *M*, pH 5.2) using an Ultra-turrax during 3 × 20 sec. Fat tissues were similarly homogenized, but in the presence of 80 ml sodium acetate buffer (0.04 *M*, pH 5.2). The pH was checked and adjusted to pH 5.2, if necessary, by using a few drops of 1 *N* HCl. After addition of 0.5 ml glucuronidase–sulphatase, hormone conjugates were hydrolysed overnight at 37°. The incubation mixture was then homogenized in the presence of 180 ml methanol and centrifuged at 10,000 *g* for 10 min using stainless-steel containers. The supernatant was decanted and fat removed by extracting the supernatant two times with 50 ml *n*-hexane. The hexane layers were discarded. The methanol phase was then extracted successively with 150, 90 and 90 ml dichloromethane. The dichloromethane phases were collected in a conical flask and evaporated to dryness on a rotary evaporator at 40°. The evaporated extract was taken up in 20 ml distilled water and percolated through an Amberlite XAD-2 column (80 × 10 mm). The evaporation flask was rinsed with 2 × 20 ml distilled water. The rinsings were allowed to percolate through the Amberlite column at 5 ml/min. The percolated extract and washings were discarded. The steroids were eluted by passing 40 ml methanol through the column. The methanol eluate was evaporated to dryness at 40°.

Extraction of hormones from urine

The analysis of anabolics from urine is shown schematically in Fig. 2. Urine (50 ml) was allowed to percolate through an Amberlite column (80 × 20 mm) at 5 ml/min. The column was washed with 100 ml distilled water. Eluates and washings were discarded. The conjugated and free steroids were eluted with 100 ml methanol. The eluate was concentrated to a volume of 1 ml in a rotary evaporator at 40°. The residue was taken up in 10 ml acetate buffer (0.2 *M*, pH 5.2) and 0.25 ml glucuronidase–sulphatase was added. A few drops of chloroform were added and the mixture was incubated overnight at 37°. The enzyme digest was diluted with 20 ml methanol and extracted with 15 ml dichloromethane. The lower phase was collected and the upper phase was reextracted two times with 10 ml dichloromethane. The pooled dichloromethane phases were evaporated to dryness at 40°.

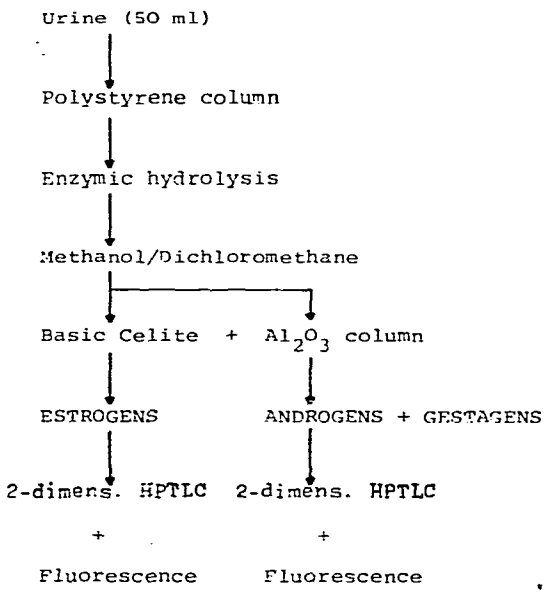

Fig. 2. Scheme for detection of anabolic steroids in urine.

Group separation of hormones

Separation of the hormones into an estrogen and non-estrogen fraction was performed in one step by passage of the extracts through a Celite–KOH column coupled to an Al_2O_3 column. The Celite column (200 × 9 mm) was mounted on top of the Al_2O_3 column so that eluate from the former passed into the latter. Meat or urine extracts were dissolved in 5 ml benzene–isooctane (1:1), quantitatively transferred to the top of the Celite column and allowed to drain into the column. The evaporation tube was rinsed twice with 5 ml benzene–isooctane and the rinsings were passed through the Celite column. After the solutions had drained into the Celite surface, the columns were uncoupled.

The Celite column was washed with 50 ml benzene–isooctane (1:1), the washings were discarded and the estrogens eluted with 50 ml water-washed diethyl ether into a conical tube, containing 1 ml absolute ethanol. The eluate was evaporated to dryness.

The Al_2O_3 column was washed successively with 25 ml benzene–isooctane (1:1) and 50 ml benzene. Washings and percolated extract were then discarded. The androgens and gestagens were eluted from the Al_2O_3 column with 30 ml benzene–ethanol (99:1) into a conical tube. The eluate was evaporated to dryness.

The dry residues, containing estrogens or androgens, were dissolved in 3 × 1 ml acetone, quantitatively transferred to a conical graduated tube (fibrinogen tube) and concentrated to near-dryness under a stream of nitrogen. The walls of the tube were rinsed with a small quantity of acetone and concentrated to 50 μl with the aid of stream of nitrogen.

Sample application

Application of small amounts of sample solution (5 nl–1 μl) was carried out by the device shown in Fig. 3. The base of the apparatus supports a slide on which the thin-layer plates (10 × 10 cm or 20 × 20 cm) rest and an arm on which a microscope micrometer and a 1/8-in. stainless-steel tubing, connected to a nitrogen cylinder, is mounted. The adjustment slide of the micrometer supports a shaft, fitting a Hamilton hand-operated dispenser with a 7001 N 1-μl syringe, needle-point style 3. By adjusting the micrometer screws the syringe may be lowered accurately to touch just the

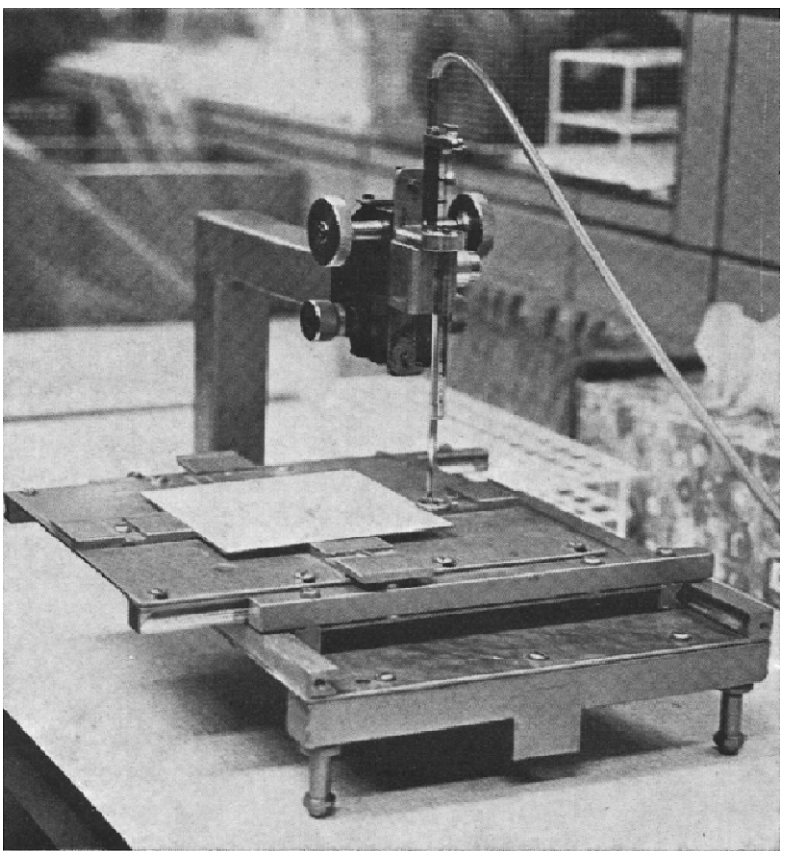

Fig. 3. Spot applicator for dispensing sample volumes at the nanolitre level.

surface of the plate. The stainless-steel tube ends in a ring (1 cm diameter) with holes of 0.25 mm drilled at regularly spaced intervals near the inside. This ring is mounted 1 cm above the thin-layer plate and centred with respect to the needle of the syringe. In this way, a jet of nitrogen may be focused on the spot of application. The slide supporting the thin-layer plates can be moved forwards, backwards or sideways, allowing the syringe to reach any point of the chromatographic plate.

Before spotting, a gentle stream of nitrogen was passed through the tube and a plate was put on the slide. The syringe was filled, slid in the shaft and lowered over the starting point so that the tip of the needle just touched the sorbent. The sample is discontinuously discharged in batches of 2 nl.

Substituting a 5-μl syringe (Hamilton 7105 N, needle-point style 3) for the syringe in the dispenser allows application of 5 μl sample solution on Nanoplates with spots measuring less than 1 mm in diameter.

Thin-layer chromatography

The extracts were analyzed by two-dimensional chromatography on precoated silica gel 60 plates. Either Nanoplates (10 × 10 cm) or 20 × 20 cm plates may be used. Development was carried out in non-saturated tanks. On Nanoplates, up to 2 μl of the extract were spotted at the starting point. Appropriate concentrations of the reference mixtures (containing 2–20 ng of the steroids presumed to be present in the sample) were applied in the side lanes. Chromatographic development was carried out, using Solvent 2, over a distance of 7 cm.

The plate was air–dried and the starting point of the sample was overspotted with 5–10 ng of the steroids presumed to be present. This procedure may be useful for additional R_F comparison between an unknown spot and the reference after the second development. The plate was then run in the second direction, using the appropriate solvent (Tables II and III).

On 20 × 20 cm plates, up to 15 μl of the extract were applied; 10–50 ng of the reference steroids were applied in the side lanes. Development was analogous to that of the Nanoplates.

Fluorescence detection. The plates were air-dried and the fluorescence reaction was induced by spraying the estrogens with 5% sulphuric acid in acetic anhydride or by spraying the androgens with 5% sulphuric acid in ethanol. The plate was viewed under UV light (366 nm) and then incubated at 95° during 12 min. The flourescence was observed under transillumination at 366 nm.

The identity of the hormones was evaluated by comparing the R_F values and the fluorescence colours of the reference substances with those of unknown spots under transillumination. Confirmation of the identity of the hormone was obtained by chromatography of the sample with a known amount of the anabolic on a second plate. After development and fluorescence reaction, the hormones in the sample must coincide exactly with the reference compounds added to the sample.

Determination of ^3H- and ^{14}C-labelled anabolics

A Packard Tri-carb 3003 liquid scintillation spectrometer was used, with toluene–methylcellosolve (4:1), containing 1.2% PPO and 0.04% dimethyl-POPOP as the scintillation fluid. Homogenized meat samples (50 g) or urine samples (50 ml) were spiked with 7×10^5 cpm of tritiated hormones or with 2×10^5 cpm of

^{14}C-labelled compounds. Samples taken at different steps in the isolation procedure were assayed as described[6].

Phase titrations

The composition of the miscibility boundary curve of dichloromethane–methanol–water was determined experimentally by using phase titration with clarification end-point[7] at ambient temperatures (18–22°). Mixtures of dichloromethane with increasing concentrations of methanol were diluted to a predetermined water content (5–95%). The phases were titrated with methanol–water mixtures of the same water content as the ternary liquids, to the disappearance of turbidity.

RESULTS AND DISCUSSION

Extraction of hormones using methanol–dichloromethane

Extraction of hormones should be quantitative for all hormones at the ppb level. Using a ternary extraction mixture, a quick separation of the layers was obtained and emulsion formation could be prevented. This avoided the time-consuming centrifugations required for diethyl ether extractions[8].

Methanol–water (3:1) allows complete removal of proteins[9] and quantitative extraction of the hormones in one step. The extraction of hormones from water–

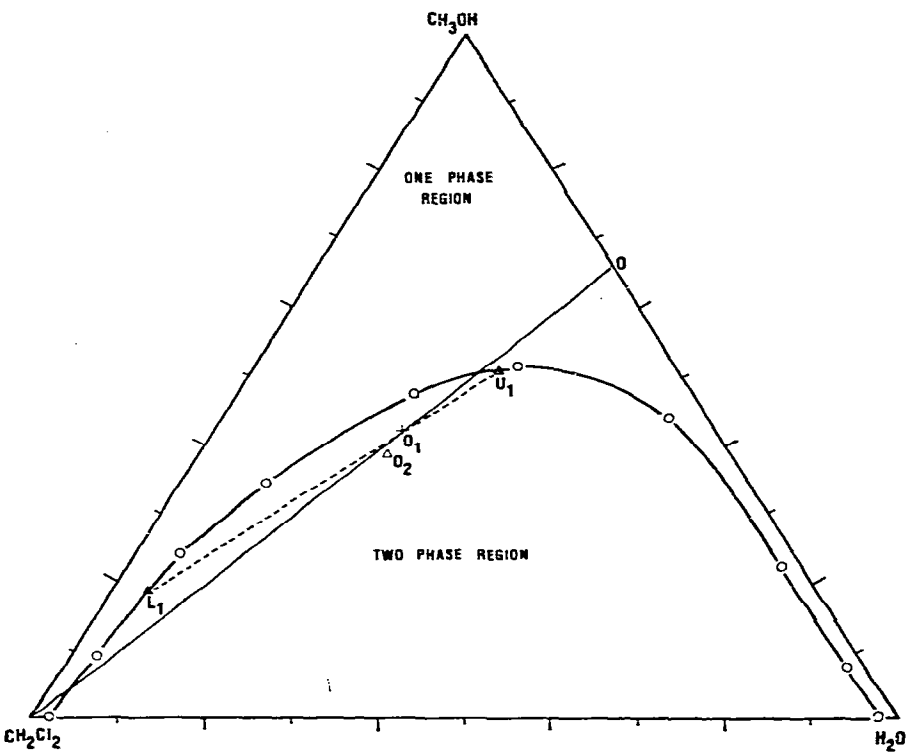

Fig. 4. Ternary liquid–liquid miscibility diagram of dichloromethane–methanol–water at ambient temperature (20°).

methanol mixtures was performed by using dichloromethane as a third solvent. Partition of hormones between the two equilibrium phases of this ternary system effects a considerable clean-up of the extracts. The optimum separation of the phases in a ternary system is obtained when the upper phase has a composition in the neighbourhood of the plait point of the miscibility curve[7] (Fig. 4). When dichloromethane is added to the water–methanol phase (1:2) (point S, Fig. 4) the composition of the mixture moves along lines DS towards point O_1 into the two-phase region. The composition of the upper and lower phases is represented by U_1 and L_1 respectively. During extraction, an amount of dichloromethane added to U_1 moves the composition of the ternary mixture towards O_2, in the neighbourhood of O_1. This procedure ensures a relatively constant composition of the equilibrium phases during repeated extractions, as is reflected by the distribution constants obtained for different anabolics (Table I).

TABLE I

DISTRIBUTION COEFFICIENTS (*D*) FOR SOME ANABOLICS IN SOME EXTRACTION SYSTEMS

D = concn. upper phase/concn. lower phase.

Solvent systems	*Testosterone*	*Trenbolone*	*Progesterone*	*DES*	*17β-Estradiol*
Hexane/67% methanol–water	0.029	0.023	0.46	0.011	0.009
67% Methanol–water/dichloromethane					
First dichloromethane extraction	0.16	0.22	0.041	0.51	0.42
Second dichloromethane extraction	0.12	0.19	0.034	0.50	0.37
Third dichloromethane extraction	0.11	0.20	0.03	0.40	0.30

Adding dichloromethane to methanol mixtures containing less than 23% water results in a tangent to the boundary miscibility curve, so that only one phase is formed. This would occur during extraction of fat tissues (< 10% water). Therefore, an extra amount of buffer is added to the fat homogenates so that a water–methanol ratio of 3:1 is obtained.

Adsorption of hormones on XAD-2 column

Anabolics as well as conjugates (glucuronides, sulphates) are quantitatively adsorbed from biological fluids by Amberlite XAD-2[5,10,11]. The recovery of the diconjugates from urine was improved when the XAD-2 resin was washed with ten column volumes of water prior to elution with methanol[10]. Solubility studies on several steroids have shown that 0.20–4.7 μg steroid may be dissolved in 1 ml distilled water[12]. Labelled steroids, in the presence of 1–100 μg of unlabelled steroids, were dissolved in 20 ml distilled water and adsorbed on an Amberlite XAD-2 column (200 × 10 mm). Adsorption of the steroids studied was quantitative, as was the elution (recovery 98 ± 1.5%; $n = 50$) with three column volumes of methanol.

Separation of anabolics into an estrogen and a non-estrogen fraction

Group separation of anabolics into an estrogen and non-estrogen fraction simplifies the identification of hormones. Adsorption chromatography of the hormones and selective displacement chromatography results in a considerable clean-up of the

extracts and is easily adapted to routine analysis. Adsorption chromatography of dienestrol on basic Celite columns[13] and the separation of 17-ketosteroids or progesterone on Al_2O_3[14] have been adapted to effect the necessary separation of the hormones.

Column chromatography of benzene extracts containing phenolic synthetic steroids (DES, DIEN, HEX) on Celite columns, saturated with 0.3 *N* KOH, yielded excellent recoveries. However, variable yields were observed with some natural estrogens (estrone, 17β-estradiol). The adsorption of estrogens from a less polar solvent (benzene–isooctane, 1:1) was essentially quantitative. Elution with water-washed diethyl ether resulted in a 95 $\pm$ 3% ($n = 40$) recovery of the estrogens.

Non-phenolic anabolics (*e.g.*, androgens, gestagens and corticosteroids) are readily adsorbed on neutral Al_2O_3 from benzene–isooctane solutions[14]. Washing the Al_2O_3 column with benzene removed different coloured bands from the column. Elution with three column volumes of benzene–ethanol (99:1) yielded quantitative recoveries (98 $\pm$ 3%; $n = 50$) of the androgens studied.

Thin-layer chromatography

Several solvent systems were tried, using standard conditions on 20 $\times$ 20 cm plates as well as on Nanoplates. The solvent combinations, used for two-dimensional chromatography (see Tables II and III), gave concentrated spots and showed a sharp separation between the different anabolics. After spraying with sulphuric acid and heating, the plates presented a dark background when transilluminated under UV light (see Fig. 5). The mobilities were measured in triplicate; standard deviations on silica gel (20 $\times$ 20 cm) plates were less than $\pm$ 0.03. Reproducibility of R_F values on Nanoplates was better than $\pm$ 0.01. Although the migration distances of estrogens and androgens were temperature-dependent[15], the relative R_F values of most anabolics were found to differ by less than 0.01 between 18 and 25°.

Sulphuric acid-induced fluorescence

Many of the reactions used for identification of steroids are based on treatment of the plates with various concentrations of acids and heating. Sulphuric acid is known to be highly selective in producing characteristic colours and fluorescence responses to minor alterations in steroids. Sulphuric acid reagents allow detection of 10–100 ng of individual steroids [16,17]. However, the fluorescence produced depends strongly on a combination of heating time and oven temperature. Therefore, the sulphuric acid-induced fluorescence was optimized after studying the responses of different sulphuric acid sprays on different hormones in combination with different heating times and oven temperatures.

Mixtures containing several estrogens (*e.g.*, DES, HEX, estrone, estradiol) or androgens (*e.g.*, testosterone, methyltestosterone, progesterone and trenbolone) were applied to plates in four concentrations. In selecting the spray reagents, the plates were heated at 95% during 10 min and the brightness of the spots, contrasts with background and differences in fluorescence colour among the various anabolics under transilluminated UV light at 366 nm were noted. Most estrogens produced brighter colours when 5% sulphuric acid in anhydrous acetic acid was used, whereas for the androgens better results were obtained by using 5% sulphuric acid in ethanol. With the spray reagents used, the optimal oven temperature for most steroids was 95° for

TABLE II

R_F VALUES, COLOURS IN UV AND DETECTION LIMITS OF SOME ESTROGENS ON THIN-LAYER PLATES AFTER SULPHURIC ACID-INDUCED FLUORESCENCE

Solvent systems: 1 = *n*-hexane–diethyl ether–dichloromethane (4:3:2); 2 = chloroform–ethanol–benzene (36:1:4). Abbreviations: BE = blue; BN = brown; BT = bright; GN = green; LT = light OE = orange; PU = purple; RD = red; YW = yellow.

Substance	*R_F value in solvent*		*Colour in UV (366 nm)*	*Detection limit*	
	1	*2*		*on plate (ng)*	*in meat (μg/kg)*
Benzestrol	0.48	0.17	GY	5	3
Dienestrol	0.53	0.18	RD	2	0.5
trans-Diethylstilboestrol	0.60	0.22	RD	1	0.5
cis-Diethylstilboestrol	0.34	0.08	RD	1	0.5
Hexestrol	0.55	0.19	GN	10	2
17α-Estradiol	0.38	0.26	YW-BT	2	0.5
17β-Estradiol	0.33	0.22	YW	3	1
17β-Estradiol benzoate	0.48	0.43	YW-BT	2.5	1
17β-Estradiol dipropionate	0.10	0.92	YW-BT	2.5	1
Estriol	0.01	0.02	YW-BN	10	2
Estrone	0.59	0.39	YW	10	2
Ethinylestradiol	0.50	0.28	YW	12	4
Equilenin	0.56	0.34	OE	2.5	1
Mestranol	0.76	0.59	YW	40	8
Zearalenone	0.50	0.30	GN-YW	5	3
Zeranol	0.17	0.18	GN-YW	5	3

10 min. Detection limits were evaluated after two-dimensional development of Nanoplates with four concentrations of hormones by using the anti-diagonal technique of Beljaars *et al.*[18]. Optimal activation of most steroids was observed at 366 nm with fluorescence maxima between 490 and 650 nm. The detection limit was set at a concentration two times the perceptible level. The results obtained with some steroids after two-dimensional development are recorded in Tables II and III. Generally, detection on 20 × 20 cm plates is five times less sensitive than on 10 × 10 cm plates.

The characteristic fluorescence colours produced by sulphuric acid, in combination with two-dimensional TLC analysis, permit specific and dependable detection of 0.2–10 ng of anabolics on the plates. The fluorescence response of sulphuric acid with most anabolics studied was more sensitive than the one obtained with 50% or 15% (w/w) phosphoric acid or with 20% (w/w) *p*-toluenesulphonic acid in ethanol[16]. DES and DIEN produce a red fluorescence (max. > 620 nm) not shown by other substances. Characteristically, DES shows up as two spots (Fig. 5): the fast running intense spot is *trans*-DES while the second fainter spot is due to *cis*-DES. The detection limit for DES is at least ten times lower that that found with the method described by Schuller[19] while the reaction is equally specific. Trenbolone or trenbolone acetate may be specifically detected by its blue fluorescence, observed immediately after spraying. Since other steroids do not react, 0.1 ng of trenbolone is easily detected, thus avoiding the use of special spray solutions described by several authors[20,21].

During preliminary work, test plates were scanned with a Zeiss TLC-scanner,

TABLE III

R_F VALUES, COLOURS IN UV AND DETECTION LIMITS OF SOME ANABOLICS OF THE "ANDROGEN" FRACTION ON THIN-LAYER PLATES AFTER SULPHURIC ACID-INDUCED FLUORESCENCE

Solvent systems: 2 = chloroform–ethanol–benzene (36:1:4); 3 = *n*-hexane–dichloromethane–ethyl acetate (1:2:2). For abbreviations see Table II.

Substance	*R_F value in solvent*		*Colour in UV (366 nm)*	*Detection limit*	
	2	*3*		*on plate (ng)*	*in meat (μg/kg)*
Chlormadinone acetate	0.60	0.74	OE	4	2
1-Dehydrotestosterone	0.29	0.28	OE-BN	5	3
6-Dehydrotestosterone	0.32	0.39	GN	2.5	1
6-Dehydroprogesterone	0.63	0.71	GN	2.5	1
5-Dihydrotestosterone	0.41	0.61	BE-PU	2.5	2
Ethisterone	0.43	0.69	YW-BN	20	5
Fluoxymesterone	0.10	0.11	BN-LT	5	5
Lynestrenol	0.69	0.94	GN	25	10
Medroxyprogesterone	0.36	0.60	BE-PU	2	1
Medroxyprogesterone acetate	0.58	0.76	YW	2.5	2
Megestrol acetate	0.57	0.74	YW-GN	2.5	1
Melengestrol acetate	0.55	0.74	YW-BN	2.5	1
Methandriol	0.28	0.43	BE-PU	2.5	2
16α-Methylprogesterone	0.65	0.76	BE-GN	5	2
16β-Methylprogesterone	0.64	0.79	BE-GN	50	10
17α-Methyltestosterone	0.36	0.48	YW-GN	1	0.5
4,9,11-Methyltestosterone	0.37	0.49	YW-GN	2.5	1
Norethinodrel	0.50	0.82	YW-LT	25	10
19-Norethisterone	0.39	0.64	BN-LT	20	10
19-Nortestosterone	0.31	0.37	YW-LT	2	2
Progesterone	0.58	0.76	BE-GN	20	10
Δ^1-Progesterone	0.49	0.63	BN-LT	10	10
Testosterone	0.32	0.43	YW	2	0.5
Trenbolone	0.31	0.38	BE-BT	1	0.2
Trenbolone acetate	0.63	0.76	BE-BT	3	0.2
Vinyltestosterone	0.43	0.67	YW-GN	2.5	1

operated with a mercury lamp, with excitation at 366 nm and a cut-off filter at 560 nm. A linear relationship between the concentration of anabolics (DES, methyltestosterone, testosterone, trenbolone) and fluorescence was found[22] in the range 1–20 ng. However, the fluorescence measured depends on the solvents used during development. Quantitative determination of these hormones after two-dimensional TLC involves an adaptation of the anti-diagonal technique described by Beljaars *et al.*[18].

Analysis of extracts from animal tissues or urine

The recoveries of labelled hormones, added in concentrations of 5–50 ppb to 50 g of tissue, were determined (Table IV). No difference in reproducibility was observed between samples prepared from muscle tissues, fat, liver or kidney. The overall recoveries are high for androgens and estrogens, but consistently lower for progesterone. The lower yields with progesterone are due to partial extraction with

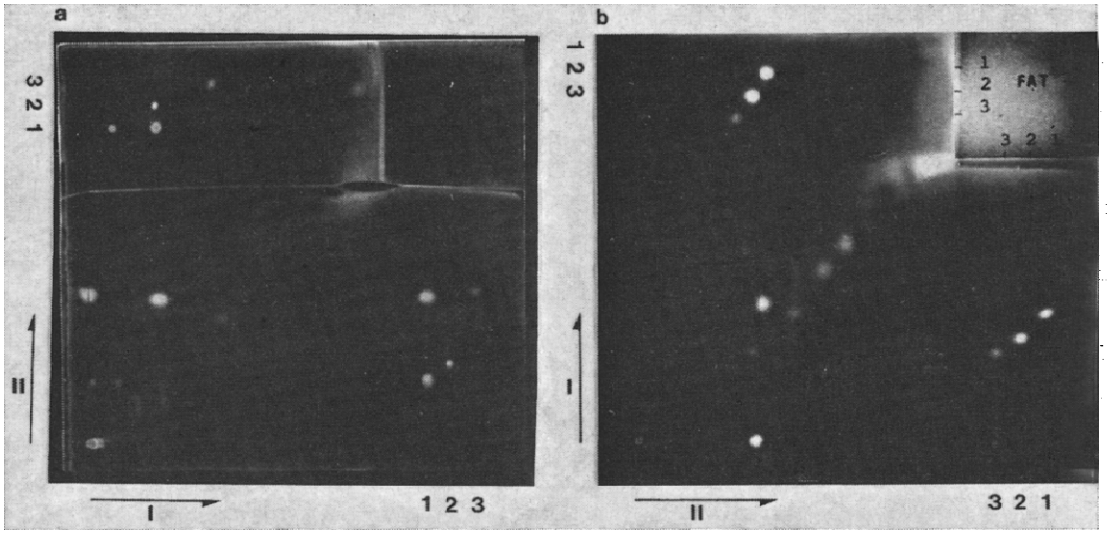

Fig. 5. (a) Two-dimensional thin-layer chromatogram on silica gel 60 (10 × 10 cm) of a fat extract contaminated with 1 ppb DES. Starting point overspotted with 2.5 ng DES before development in second direction. References in side lanes: 1 = 2.5 ng DES; 2 = 10 ng 17β-estradiol; 3 = 20 ng oestrone. Developing solvents: I = chloroform–ethanol–benzene (36:1:4); II = *n*-hexane–diethyl ether–dichloromethane (4:3:2). (b) Two-dimensional thin-layer chromatogram on silica gel 60 (10, × 10 cm) of a fat extract contaminated with 2 ppb 17α-methyltestosterone. Starting point overspotted with 3 ng 17α-methyltestosterone before development in second direction. References in side lanes: 1 = 3 ng 17α-methyltestosterone; 2 = 3 ng testosterone; 3 = 2 ng trenbolone. Developing solvents: I = chloroform–methanol–benzene (36:1:4); II = *n*-hexane–dichloromethane–ethyl acetate (1:2:2).

n-hexane during fat removal (Table I). The recoveries obtained with this extraction procedure compare favourably with results found with other TLC assays after spiking meat with 10–50 ppb trenbolone (63.5 ± 14%)[23], 17β-estradiol (38 ± 13%)[24] or DES (30%)[25].

From Table IV it is evident that the overall recovery of anabolics can be significantly increased, since *ca.* 80% of the added androgen or estrogen radioactivity was recovered in the eluates of the Al_2O_3 or Celite columns. The significantly

TABLE IV

OVERALL RECOVERY OF ^{14}C- OR ^{3}H-LABELLED ANABOLICS ADDED TO MEAT OR KIDNEY FAT

n = Number of individual experiments.

Extraction step in procedure	*Recovery (%) (mean ± S.D.)*		
	[^{3}H]trenbolone or [^{14}C]testosterone (n = 16)	*[^{14}C]progesterone (n = 14)*	*[^{3}H]DES or [^{14}C]estradiol (n = 24)*
After extraction with dichloromethane	92.2 ± 3.1	83.0 ± 4.1	91.8 ± 2.6
After XAD-2 column adsorption	91.6 ± 4.2	79.6 ± 4.8	90.7 ± 2.7
After Celite column adsorption			81.6 ± 2.4
After Al_2O_3 column adsorption	82.6 ± 5.2	74.4 ± 5.1	
Concentrate	71.3 ± 6.3	65.9 ± 5.2	70.7 ± 4.0

lower yields in the concentrated extracts must be due to partial adsorption of the anabolics on the glass wall during evaporation of the extracts to a small volume. Recently, higher recoveries for androgens and estrogens were obtained (± 75%) by substituting methanol for acetone during concentration of the extracts.

Labelled anabolics, added in concentrations of 5–50 ppb (μg/l) to urine, were recovered in excellent yields through the entire extraction and clean-up procedure (Table V). However, concentration of the extracts led to drastic decreases in recovery. As with meat samples, recoveries up to 75% have been obtained recently by substituting methanol for acetone.

TABLE V

OVERALL RECOVERY OF ^{14}C- OR ^{3}H-LABELLED ANABOLICS ADDED TO URINE

n = Number of individual experiments.

Extraction step in procedure	*Recovery (%) (mean ± S.D.)*		
	[^{3}H]trenbolone or [^{14}C]testosterone (n = 10)	*[^{14}C]progesterone (n = 13)*	*[^{3}H]DES or [^{14}C]estradiol (n = 12)*
After XAD-2 column adsorption	95.2 ± 1.3	99.5 ± 1.6	97.1 ± 2.1
After extraction with dichloromethane	92.4 ± 1.3	97.2 ± 2.0	90.5 ± 5.4
After Celite column adsorption			80.6 ± 3.0
After Al_2O_3 column adsorption	78.4 ± 3.0	90.9 ± 3.6	
Concentrated extract	64.8 ± 3.6	77.5 ± 5.5	49.1 ± 4.0

In series of experiments, blank samples of meat were spiked with hormones to a final concentration of 0.5–20 ppb. Following extraction and clean-up, approximately one tenth of the extract was analyzed by two-dimensional TLC. After sulphuric acid-induced fluorescence, the presence of the hormones on the plates was detected by the characteristic fluorescence colours of the substances and the relative mobilities of the spots. The detection limits for several hormones in the presence of meat or tissue extracts are recorded in Tables II and III. As shown in Fig. 5, muscle, tissue and fat extracts (except liver) were free from interfering substances: concentration of the extract to smaller volumes allowed even lower detection limits to be reached than those given in the tables. The detection limits of residues in tissues do not always parallel the limits obtained with reference solutions on the chromatographic plate. Spot-broadening during development, contrast of the spots against the background and separation from interfering spots may be even more important. This is observed when urine or liver extracts are analyzed: diffuse spots result in detection limits twice as high as those observed for meat or fat.

Use of this procedure in the control of anabolic residues in animal tissues and urine

The method described has been accepted by the Belgian Ministry of Public Health for the control of anabolic residues in slaughtered animals and in urine. The procedure has been in routine use in three different laboratories during at least 2 years. One analyst can handle at least ten complete residue analyses per week. Pretreatment, extraction, clean-up and concentration of five samples takes *ca.* 1.5 working days. TLC analysis of the estrogen and androgen fractions and, if necessary,

co-chromatography of the samples in presence of the suspected hormones may require another working day.

More than 600 samples of urine, muscle tissues, fat or organs from slaughterhouse animals have been analyzed in our laboratory over the last 3 years, using this method. In urine samples, residue levels of 2–40 ppb of various anabolics were often found. The residue concentrations detected in meat and fat were lower and rarely exceeded 5 ppb. However, it would be hardly expected that residue levels in muscle tissues would reach the detection limit of this method, if the animals were dosed properly and an adequate time interval before slaughtering was allowed[2,4]. Therefore, efforts were undertaken to improve the efficiency of the surveillance by sampling the tissues of the carcass with the highest residue concentration.

Recently, the distribution of anabolic residues among different tissues and organs of animals treated with methyltestosterone and diethylstilboestrol have been studied[26]. Compared with most muscle tissues (*e.g.*, *Musculus longissimus dorsi*, *M. gracilis*) a two- to five-fold higher residue concentration was found in liver, kidney, diaphragm muscle or most fats. Similar findings were reported earlier for trenbolone, estrone and testosterone[2]. However, the residue levels in kidney fat were consistently higher than those observed in most fats and amounted to 5–20 times the concentrations observed in *M. longissimus dorsi*. When kidney fat was used as a marker tissue, residue levels of at least 1 ppb have been found after treatment of heifers or steers with normal doses of diethylstilboestrol and methyltestosterone (see Fig. 5).

To be used with success in the regulatory control of the abuse of anabolics, methods should thus have an adequate sensitivity (1–10 ppb) and specificity, allowing the simultaneous detection of various combinations of anabolics frequently encountered in the illegal practice. What has often been overlooked is that TLC analysis of hormones coupled with group selective reagents (*e.g.*, Dansyl derivatives of estrogens[24,27–29] and androgens[29], or azo dyes coupled to estrogens[30,31]), although potentially extremely sensitive, in practice lacks the desired specificity. Because extracts of meat or urine contain substances reacting with Dansyl reagents, a multitude of different spots appear on the TLC plate[4]. Owing to interfering background spots, the practical detection limit of the anabolics in animal tissues and urine is raised to at least 10 ppb when group-selective reagents are used.

CONCLUSIONS

A routine method is described for the simultaneous detection of various anabolic residues in animal tissues or urine at levels of 0.5–10 ppb.

The extraction and clean-up procedure permits adequate recovery (60–80%) of the anabolics added to tissue or urine samples. Two-dimensional TLC analysis combined with sulphuric acid-induced fluorescence allows specific detection of 0.5–10 ng of individual anabolic residues on the plates.

The major advantages of this method over other TLC methods and RIA lie in its general applicability to several anabolics, high sensitivity, and reproducibility.

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