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General and selective isolation procedure for high-performance liquid chromatographic determination of anabolic steroids in tissues

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ABSTRACT

A multi-residue method has been developed for the determination of anabolic steroids in animal tissue. The analytes are extracted from tissue with methanol and the extract is subjected to two solid-phase extractions, one using a non-specific adsorbing material, such as graphitized carbon black (Carbopack B), and the other Amberlite CG-400 I in the OH form. This procedure allowed the neutral anabolics (testosterone, trenbolone and progesterone) to be isolated and separated from the acidic type (phenolic group), such as diethylstilbestrol, oestradiol, zeranol/zearalenone and their respective metabolites. The determination was effected using high-performance liquid chromatography with different detectors (ultraviolet, fluorimetric and electrochemical). Several analytical parameters were studied: chromatographic conditions, recoveries, evaporation step, solvent flow-rate, cartridges reusability, interference of plastic cartridges. For all the anabolics investigated the recoveries were >83.6%.

INTRODUCTION

Anabolic compounds stimulate synthesis and thus increase the muscle size and strength in both humans and animals. Anabolic substances are used in animal production (cattle and sheep) to enhance rapid growth and to improve feed efficiency, resulting in economic benefits to both farmer and consumer. The compounds found to be effective in humans and animals are invariably steroids structurally related to the natural androgen testosterone, whereas in cattle either androgenic or oestrogenic agents or combinations of the two may be used. These compounds may be derived either from the natural steroids or from synthetic steroids, *e.g.*, oestradiol, testosterone, progesterone or trenbolone, or else may have a non steroid structure, *e.g.*, diethylstilbestrol, zeranol.

Improper use of these hormones or too short a withdrawal period between treatment and slaughter of the animals may result in high residual levels in the edible portion of the treated animals. The use of anabolics in animal feeds involves possible health risks if harmful residues remain in the meat products intended for human consumption [1].

At present, certain anabolic compounds can be legally given to farm animals in some countries but are banned in others. For example, the European Community (EEC) [2] has banned the use of all anabolic steroids because of their proven or alleged toxic and/or carcinogenic properties. Therefore the necessity to test for illegal use or to determine residue levels after legal use has led to a strong interest in methods for the detection of anabolizing agents in biological samples and in food products. Numerous techniques for determining these oestro-

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genic compounds and their metabolites have been reported.

The most frequently used techniques for the dosages concerned involve either chromatographic [thin-layer, high-performance liquid (HPLC) and gas chromatography (GC) and GC–mass spectrometry] [3–14] or immunochemical detection [15–18]. Many of these methods are tedious or comparatively insensitive. Immunoassays are highly sensitive, but analysis is limited to a single specific compound against which the antibody was raised and non-specific binding of a steroid antibody tends to produce false-positive results.

In analyses for single or multi-component anabolic compounds HPLC has proved to be an excellent separation, purification and detection technique. Ryan [19] reviewed its use in the analysis of hormone residues, including diethylstilbestrol (DES), oestradiol and zeranol. Other investigators have also reported the determination of zearalenone and α-zearalenol in porcine plasma, in chicken fat, heart and kidney and in animal feed. Groham *et al.* [20] developed a multi-residue analysis for anabolics (oestradiol, DES, zeranol, dienoestrol, hexoestrol and ethinyloestradiol) in meat. Medina and Sherman [21] described a multi-residue analysis for avian muscle tissue. Bagneris *et al.* [22] used liquid chromatography with fluorescence detection to determine zearalenone and zearalenol in animal feeds. Roybal *et al.* [23] described a method to determine zeranol, zearalenone and their metabolites in edible animal tissue. Frischkorn *et al.* [24] reported an LC system with voltammetric detection of several growth-promoting hormones.

The objectives of this study were to develop a method to resolve the anabolic steroids and their metabolites possessing high oestrogenic activities. The general procedure proposed consists of (i) a dual-column clean-up system for a methanol extract of from the tissue, one column containing a non-specific adsorbing material, such as graphitized carbon black (Carbopack B), and the other filled with Amberlite CG-400 I in the OH form; and (ii) the development of a reversed-phase HPLC method to separate the substances investigated followed by either UV detection or more sensitive and more highly specific fluorimetric or electrochemical detection.

EXPERIMENTAL

Reagents

Fig. 1 shows the structures of the compounds investigated. The anabolic compounds used as reference materials were obtained as follows: trenbolone (17β-hydroxy-4,9,11-estratien-3-one) from LabService (Bologna, Italy), DES [4,4′-(1,2-diethyl-1,2-ethenediyl)bisphenol], testosterone (17-β-hydroxy-4-androsten-3-one), progesterone (4-pregnen-3,20-dione) and 17β-oestradiol-[1,3,5-(10)-estratiene-3, 17-β-diol] from Sigma (St. Louis, MO, USA), taleranol [6-(6,10-dihydroxyundecyl)-β-resorcylic acid μ-lactone], zearalenol [6-(6,10-dihydroxyundecyl-*trans*-1)-β-resorcylic acid and zeranol [6-(6,10-dihydroxyundecyl)-β-resorcylic acid] from International Minerals and Chemical Corp. (Terre Haute, IN, USA). Standard solutions were prepared by dissolving accurately weighed 10-mg amounts of each reference standard in 100-ml volumetric flasks in methanolic solution. The solutions were then further diluted to obtain working standard solutions. The distilled water used was further purified by passing it through a Norganic cartridge (Millipore, Bedford, MA, USA). Acetonitrile of G Chromasolv grade (minimum transmittance 75% at 195 nm, 93% at 200 nm, 95% at 210 nm and 98% at 220 nm, path length 1 cm *vs.* water) was obtained from Riedel-de Haën (Hannover, Germany) and methanol, methylene chloride and tetrahydrofuran of HPLC grade from Carlo Erba (Milan, Italy). All other chemicals were of analytical-reagent grade (Carlo Erba) and were used as supplied.

Apparatus

Glass liquid–solid extraction columns (laboratory made) (6 cm × 8 mm I.D.) were filled with Carbopack B and columns (6 cm × 4 mm I.D.) were filled with Amberlite CG 400 I, both by a dry-packing technique. The columns were fitted with a Luer-type connection so that they could be inserted into the solid-phase extraction vacuum manifold (Supelco, Bellefonte, PA, USA). Carbopack B (200–400 mesh) was kindly supplied by Supelco. Amberlite CG-400 I (100–200 mesh) was obtained from Serva (Heidelberg, Germany). Small wads of quartz-wool were inserted in the head and tail of the glass columns.

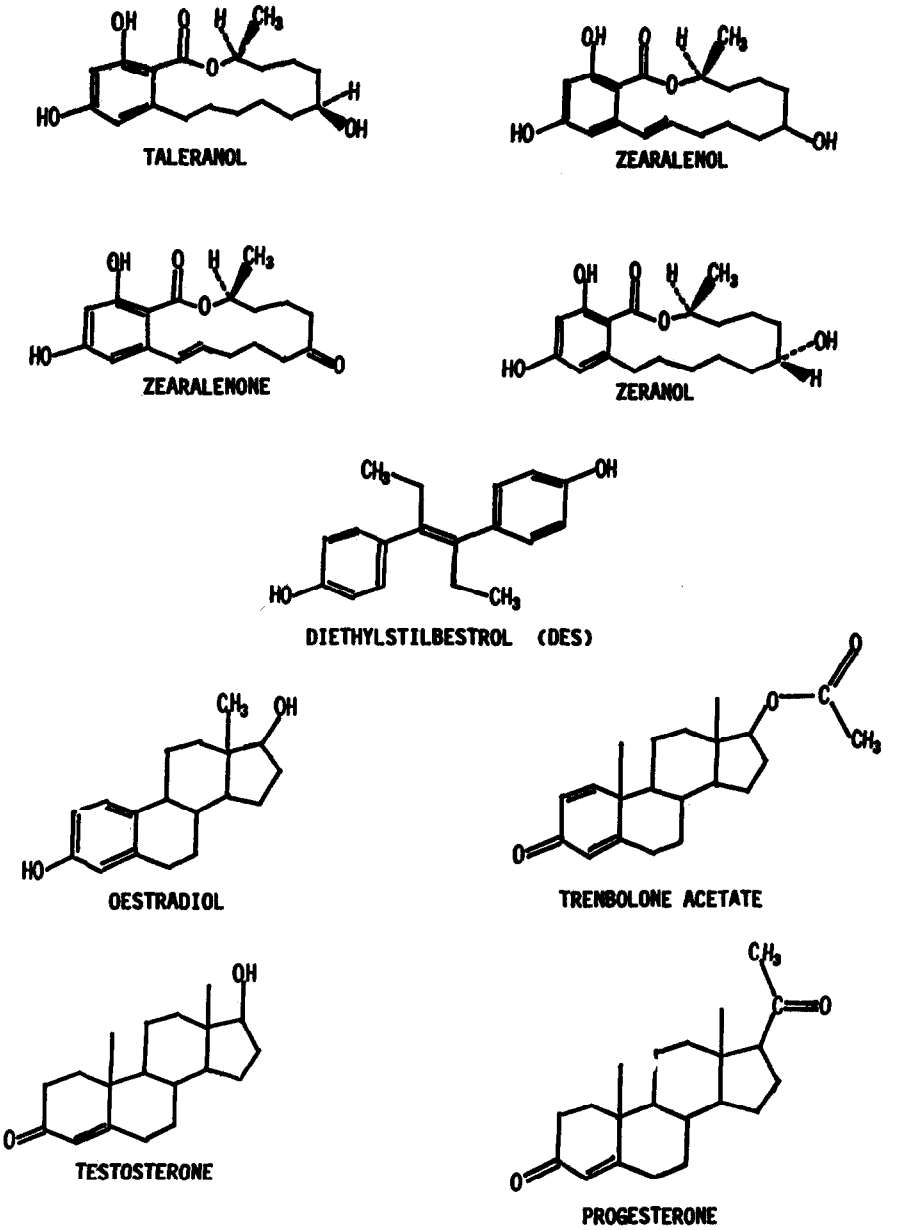


Fig. 1. Structures of compounds.

Instruments

A Series 410 liquid chromatograph (Perkin-Elmer, Norwalk, CT, USA) was used, equipped with a Rheodyne Model 7125 injection valve having a 100-μl loop, a Model LC 95 UV detector (Perkin-Elmer), a Model LS 3 spectrofluorimeter (Perkin-Elmer), and an electrochemical detector Model 5100A Coulochem (ESA, Bedford, MA, USA).

An LC-I 100 laboratory computing integrator (Perkin-Elmer) was used for peak-area measurements. A 25 cm × 4.6 mm I.D. column filled with 5-μm (average particle size) C_{18} reversed-phase

packing and a 2 cm × 4.6 mm I.D. Supelguard LC-18 (5 μm) column (both from Supelco) were used: A Polytron homogenizer (Kinematica, Kriens-LU, Switzerland) and a Uniset-ac ultrasonic bath (AGE Elettronica, Italy) were used.

Chromatographic conditions

The chromatographic conditions were selected according to the type of molecules involved and the detection technique. Acetonitrile–0.010 *M* KH_2PO_4 (pH adjusted to 3.0 with H_3PO_4) (48:52, v/v) was used for DES determination, with the electrochemical detectors set at 0.05 V (detector 1) and 0.30 V (detector 2), the flow-rate being 1.2 ml min^{-1}. The mobile phase for oestradiol determination was acetonitrile–0.010 *M* KH_2PO_4 (pH adjusted to 3.0 with H_3PO_4) (46:54, v/v). The fluorescence detector was set at λ_{ex} = 280 nm and λ_{em} = 308 nm, the flow-rate being 1.2 ml min^{-1}. For the determination of zeranol/zearalenone and their metabolites, 0.01 *M* KH_2PO_4 (pH adjusted to 3.0 with H_3PO_4)–acetonitrile–methanol–tetrahydrofuran (THF) (60:21:7:12, v/v) was used, the flow-rate being 1.5 ml min^{-1}. The electrochemical detectors were set at 0.05 V (detector 1) and 0.60 V (detector 2).

For testosterone, trenbolone and progesterone, an acetonitrile–water gradient was used, from 40% to 65% acetonitrile in 30 min, the flow-rate being 1.2 ml min^{-1}.

The absorbance of the effluent was monitored at 242 nm. In all experiments, the chromatographic column was operated at room temperature.

Quantitative data

The concentrations of anabolics in tissue samples were calculated by measuring the peak-area ratios of each oestrogenic compound to an internal standard and comparing peak-height ratios of the extracts with those of non-processed standards. The latter were prepared by adding an appropriate volume of anabolic working standard solution to an equal volume of the sample mixture used for the elution of anabolics from the clean-up column. To this solution were added 25 μl of solution containing the internal standard.

Pretreatment of column

Before use, the Carbopack B and resin columns were pretreated. The glass column was packed with 250 mg of Carbopack B and 5 ml of methanol, 15 ml of dichloromethane–methanol (70:30, v/v) and 10 ml of methanol–water (85:15, v/v) were successively passed through it. The glass columns used to be filled with resin were packed with 50 mg of Amberlite CG-400 I. The anion-exchange material was then purified and converted from the Cl^- to the OH^- form by treatment with 3 ml of 0.5 *M* sodium hydroxide solution and 8 ml of dichloromethane–methanol (70:30, v/v), then washed with 1 ml of water and finally deactived with 3 ml of 1 *M* HCl in methanol. This washing cycle was repeated four times, and finally the resin was converted into the OH^- form by passing through it 20 ml of 0.05 *M* sodium hydroxide solution and washed with 1 ml of water. When not in use, the column was kept in water.

Sample preparation, extraction and clean-up procedures

Portions of chicken (breast) muscle and ox muscle and liver were stored in a freezer. A 1-g amount of tissue was homogenized in 5 ml of methanol in the Polytron homogenizer and then sonicated for 5 min and centrifuged at 6000 rpm for 10 min (3956 *g*). Another 5 ml of methanol were added to the pellet and the extraction process was repeated and the supernates for each sample were pooled. To the remaining methanol solution, which had partially evaporated during the treatment, methanol was added to give a volume of 6.8 ml, then 1.2 ml of water were added in order to obtain 8 ml of a final solution of methanol–water (85:15, v/v). This mixture was allowed to percolate through the Carbopack B column and the eluate collected in a test-tube (I). The column was then washed with 2 ml of methanol–water (85:15, v/v), and the eluate again collected in the same test-tube (I). The column was washed with 2 ml of methanol, which was discarded.

Lastly, all the anabolizing agents except DES (already eluted into I) were eluted from the Carbopack column with 8 ml of dichloromethane–methanol (70:30, v/v) (test-tube II). The solution contained in test-tube I was allowed to percolate through one of the Amberlite columns, after which the column was washed with 1 ml of methanol and 1 ml of 1 *M* HCl (aspirating for 1 min in a vacuum after this last step). The DES was eluted into conical silanized tubes with 2 ml of 0.03 *M* HCl in acetoni-

trile–methanol (20:80, v/v). The eluate was evaporated to dryness using nitrogen at 40°C. The dried eluate was then immediately taken up with 100 μl of acetonitrile–0.010 *M* KH_2PO_4 (pH adjusted to 3.0 with H_3PO_4) (48:52, v/v) and a 50-μl portion was injected.

The solution contained in test-tube II was passed through the second resin-filled column, collecting the eluate in a silanized conical tube (III). The column was washed with 1 ml of methanol, added to tube III, and with 1 ml of 1 *M* HCl, which was discarded. The acidic anabolics (taleranol, zearalenol, zeranol, zearalenone and oestradiol) were eluted into silanized conical tube with 2 ml of 0.03 *M* HCl in acetonitrile–methanol (20:80, v/v). This eluate was evaporated to dryness with nitrogen at 40°C. A 100-μl portion of 0.010 *M* KH_2PO_4 (adjusted to pH 3.0 with H_3PO_4)–acetonitrile–methanol–THF (57:22:8:13, v/v) was added and a 50-μl portion was injected for the determination of zeranols. A 40-μl portion of the remaining amount was injected for the determination of oestradiol.

The solution of neutral anabolics contained in tube III was evaporated to dryness under nitrogen at 40°C, then taken up with 200 μl of methanol–water (50:50, v/v); 25 μl of *p*-chlorophenol (10 ng/μl) were added as internal standard (I.S.) and a 50-μl portion was injected.

RESULTS AND DISCUSSION

In biological fluids, anabolic steroids are present in a very complex matrix of other compounds, some of which are structurally very similar. Correct determinations of concentration can be achieved only by a combination of extensive purification of the sample and by using sensitive and specific detection systems. The main anabolizing agents are neutral or acidic compounds (phenolic group). Utilization of differences in functional groups may therefore considerably simplify the purification procedure needed for HPLC analysis.

Extraction of anabolic steroids from tissue

Extraction of anabolic steroids should be quantitative for all hormones at the ng/g level. The quantitative extraction of anabolic steroids from tissue without the formation of artefacts is extremely difficult, as isolation by means of solvents does not give a true picture of the extraction of the molecules present in the tissue. For this purpose, solvents must be used that have been shown to extract a wide range of lipids quantitatively and which are good solvents for anabolic steroids.

Methanol allows the complete removal of proteins [25] and quantitative extraction of hormones. Verbek [4] used a ternary system for the extraction of anabolics. Chloroform–methanol has been extensively used for the extraction of lipids from tissue [26], while Andersson and Sjovall [27] used *n*-hexane–isopropyl alcohol to isolate steroids from tissue. In comparative studies, Hara and Radim [28] also showed that most lipids can be quantitatively extracted with *n*-hexane–isopropyl alcohol.

As a preliminary measure, therefore, the extraction system must be selected on the basis of the solvent or solvent mixture which provides the highest relative extraction efficiency. For this purpose it is particularly important to use solvents that are capable of interacting with both the hydrophilic and hydrophobic components of the tissues. We investigated three solvent systems: methanol, methanol–chloroform and *n*-hexane–isopropyl alcohol (60:40, v/v). Comparative studies carried out on portions of animal tissues treated with the above substances showed that all three systems display comparable extraction percentages and therefore comparable extraction efficiencies. For our investigation we chose methanol, as it can be directly diluted with water in the ratio 85:15. This phase, which contains the extracted anabolic steroids, can then be percolated through the Carbopack B column, thus ensuring total adsorption of the dissolved substances. Because of their lower polarity, during Carbopack B adsorption the other two extraction systems allow only partial retention of these molecules. The problem can be solved by drying and dissolution in highly polar mixtures [methanol–water (85:15, v/v)], although the evaporation operation means a further loss of time. The main steps in the procedure during the analysis of anabolics from animal tissues are summarized in Fig. 2.

Adsorption of anabolic steroids on Carbopack B and Amberlite columns

The combination of an extraction trap containing a non-specific adsorbing material and the subsequent specific sorption of acidic compounds using a

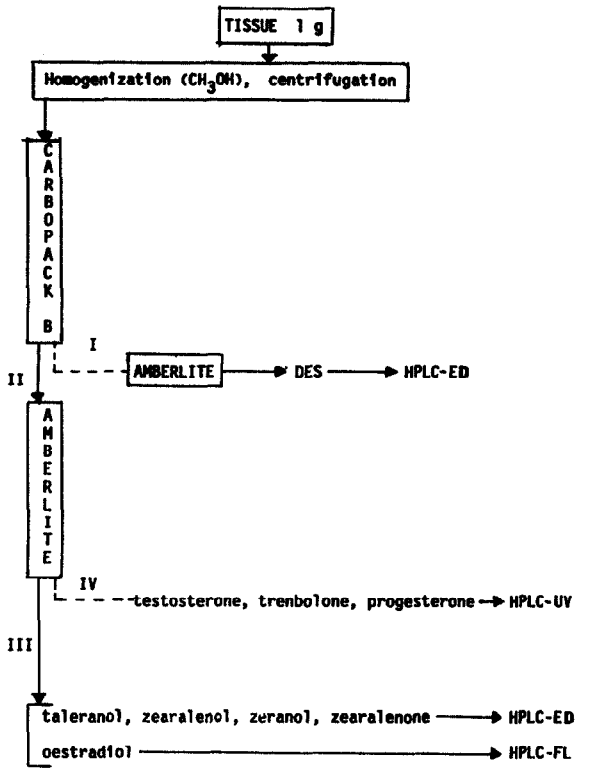
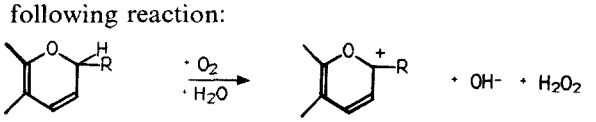


Fig. 2. Scheme of analytical procedure. Numbers I, II, III and IV relate to the various fractions eluted from extraction columns. ED = Electrochemical detection; FL = fluorimetric detection.

column filled with an anion exchanger allows us to simplify the isolation of the analytes and the subsequent identification of the anabolic steroids by means of multi-component analysis.

Acidic compounds display a typical behaviour on the Carbopack B surface. This effect can be explained by assuming that the Carbopack B surface is contaminated by certain positively charged chemical impurities, which may be a burnt-off residue left over from heating at 2700–3000°C of carbon black. Recently, we obtained experimental evidence [29,30] for the existence of a chromene-like structure in the surface framework of Carbopack B. This oxygen complex in the presence of oxygen and water can be rearranged to form a structure similar to benzopyran and benzopyrylium salts according to the following reaction:



With acidic molecules, these salts are the cause of very strong interactions and so their elution requires solvent mixtures acidified with HCl [31,32]. However, recovery phases containing Cl^- ions are not compatible with our second extraction trap, because sorption is prevented from occurring as the Cl^- concentration has saturated the resin. Consequently, care has to be taken to avoid the formation of benzopyrylium salts.

Before use, the Carbopack B column is treated with non-aqueous solvents and solvent mixtures in order to avoid the formation of these salts. A partially aqueous solution [methanol–water (85:15, v/v)] does not allow these salts to be formed. All the anabolic steroids contained in the methanol–water (85:15, v/v) phase except DES are quantitatively adsorbed on the Carbopack B surface. Under conditions of percolation through the Carbopack B column the phenolic synthetic steroid (DES) is not adsorbed because its polarity exceeds that of the other anabolics. The solvent mixture dichloromethane–methanol (70:30, v/v) used to elute the anabolics quantitatively from the extraction column is also an ideal medium for their complete readsorption on the second isolation column (Amberlite).

Isolation of anabolics with specific functional groups on an exchanger converted to a suitable form is, in a sense, comparable to affinity chromatography. The only anabolics that can be sorbed on a resin in the OH^- form are those which can form complexes of the AOH^- type, where A indicates a generic acid molecule, whereas natural steroids (testosterone, trenbolone and progesterone) are not sorbed and therefore pass through the resin and can be analysed separately. Desorption of anabolic steroids from Amberlite was achieved by using acetonitrile–methanol (20:80, v/v), acidified with 0.03 *M* HCl.

The chloride ion has proved very effective in displacing the AOH^- complex from exchanger sites. It has been observed that if washing with 1 *M* HCl is omitted from the desorption operation, most of the non-exchanged resin sites will still be in the OH^- form. Therefore, these ions can enter into specific interactions with the phenol ions (hydrogen-type bond), thus extending the elution range. This behaviour has been tested experimentally by having the anabolic steroid mixture sorbed directly at the head of the Amberlite column.

In the procedure involving washing with 1 *M* HCl it was found that all the anabolics were eluted from the recovery mixture using only a 2-ml volume. On the other hand, in order to achieve quantitative recoveries, about 20 ml of acetonitrile–methanol (20:80, v/v) acidified with 0.03 *M* HCl were required.

Recoveries and reproducibility studies

Recoveries were initially performed on commercially available chicken breasts (muscle), ground beef (muscle) and ox liver. The samples were spiked with anabolics to a final concentration of 1–20 ppb (ng/g). Known amounts of hormones were added to the tissue solvent homogenate. The entire clean-up procedure and subsequent quantification were then performed. Recovery was calculated by subtracting the concentration of the endogenous steroid from the concentration of the steroid contained in the tissue to which it had been added; the difference was divided by the peak heights for the same spike dissolved in the final volume. The analytical recovery and the accuracy of the method at high (20 ng/g) and low (1 ng/g) muscle homogenate steroid contents were evaluated. In all instances the recoveries were at least 91.4 ± 4.1% for all substances except DES, for which it was at least 83.8 ± 4.0%. No difference in reproducibility was observed between samples prepared from different types of muscle tissue.

Chromatographic experiments

The chromatographic conditions were chosen on the basis of the function of the substances to be analysed and the detection technique used.

Optimum conditions for the separation of neutral compounds were found to be gradient elution from the acetonitrile–water (65:35, v/v) mobile phase in 30 min at a flow-rate of 1.2 ml min^{-1}; the peaks were detected at 242 nm. The chromatograms referring to the standard testosterone, trenbolone and progesterone mixture and a typical chromatogram referring to beef (muscle) to which the above anabolics had been added are shown in Fig. 3a and b. The type of acetonitrile used in the gradient elution is particularly important, because even when acetonitrile was used for HPLC the baseline was raised for high sensitivities. A gradient acetonitrile, "acetonitrile G", has recently come onto the market which minimizes this effect. For the separation of zeranol, zearalenone and their respective metabolites, iso-

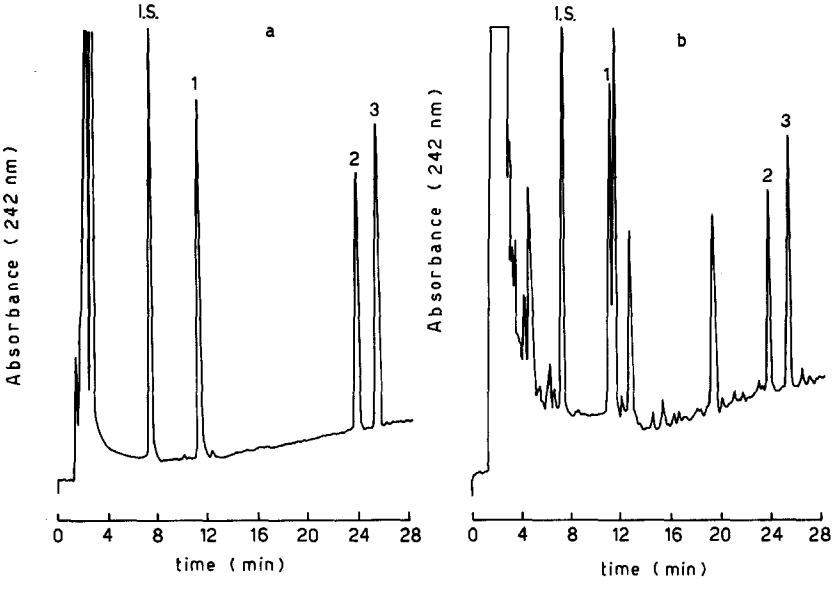


Fig. 3. HPLC separation of steroids with gradient elution and UV detection. Mobile phase, linear gradient of acetonitrile–water from 40% to 65% acetonitrile in 30 min; flow-rate, 1.2 ml min^{-1}; UV detection at 242 nm. (a) Standard mixture: I.S. = *p*-chlorophenol; 1 = testosterone; 2 = trenbolone; 3 = progesterone. (b) Chromatogram of extracted beef muscle spiked with (I.S.) *p*-chlorophenol, (1) testosterone (19 ng/g), (2) trenbolone (28 ng/g) and (3) progesterone (19 ng/g).

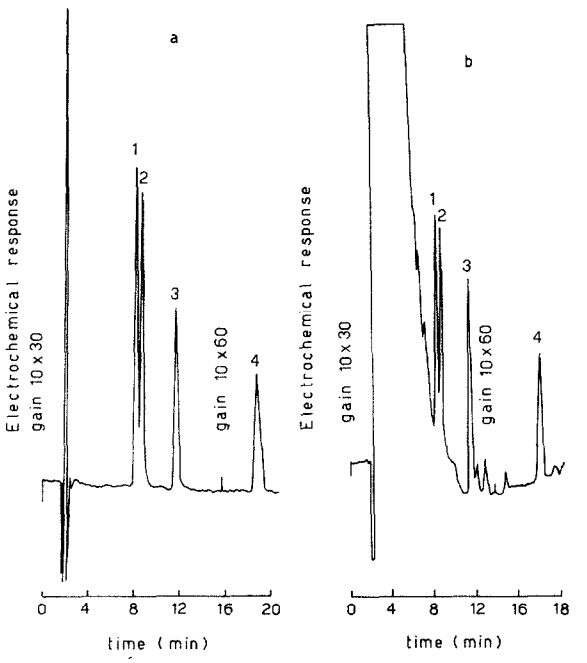


Fig. 4. HPLC of anabolics with isocratic elution and electrochemical detection. Mobile phase, 0.01 *M* KH_2PO_4 (adjusted to pH 3.0 with H_3PO_4)–acetonitrile–methanol–THF (60:21:7:12, v/v); flow-rate, 1.5 ml min^{-1}; electrochemical detection, detector 1 at 0.05 V, detector 2 at 0.60 V. (a) Standard mixture: 1 = taleranol; 2 = zearalenol; 3 = zeranol; 4 = zearalenone. (b) Chromatogram of extracted chicken muscle spiked with (1) taleranol (12 ng/g), (2) zearalenol (16 ng/g), (3) zeranol (13 ng/g) and (4) zearalenone (12 ng/g).

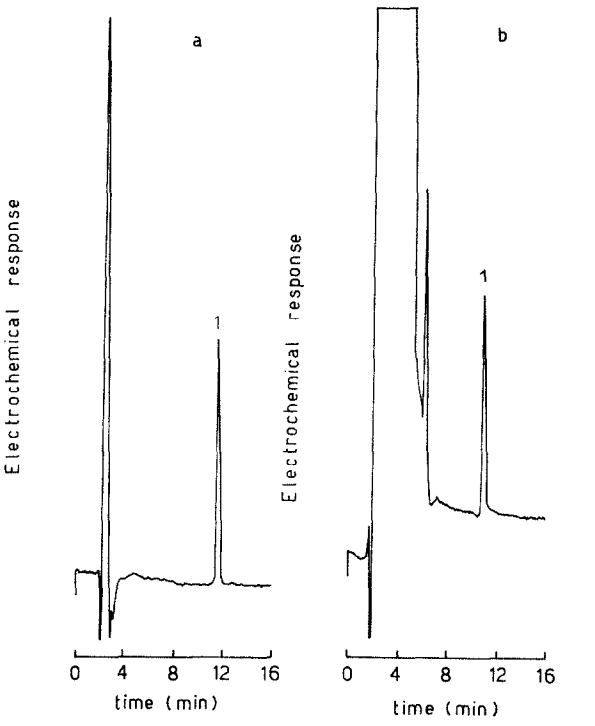


Fig. 5. HPLC of DES with isocratic elution and electrochemical detection. Mobile phase, 0.01 *M* KH_2PO_4 (adjusted to pH 3.0 with H_3PO_4)–acetonitrile (52:48, v/v); flow-rate, 1.2 ml min^{-1}; electrochemical detection, detector 1 at 0.05 V, detector 2 at 0.30 V. (a) Standard solution: 1 = DES. (b) Chromatogram of extracted beef muscle spiked with (1) DES (6 ng/g).

cratic elution with electrochemical detection is used.

Frischkorn *et al.* [24] used acetonitrile–water with LiCl and $LiClO_4$, which does not separate *cis*-zearalenone from *trans*-zearalenone. Roybal *et al.* [23] used methanol–sodium acetate, although this phase does not separate zearanol from taleranol. Our mixture completely separates taleranol from zeranol on the baseline and almost completely separates taleranol from zearalenol. The retention of these compounds is highly dependent on the acetonitrile and THF content. By maintaining the percentage of water constant and varying the percentage of organic solvents, the behaviour of these compounds was observed to vary. At higher percentages of tetrahydrofuran an enhanced separation between taleranol and zearalenol was obtained, although the zearalenone was eluted with a long retention time (> 30 min). An acceptable compromise was reached at higher acetonitrile percentages, although an unknown peak interfering with zeranol was found with tissue samples. The best mobile phase for separating the unknown substance from zeranol was found to be that reported under Experimental. The separation of a standard mixture of taleranol, zearalenol, zeranol and zearalenone is shown in Fig. 4a, and a chromatogram relating to the extract of a chicken tissue to which a mixture of zeranol and zearalenone and their respective metabolites had been added is shown in Fig. 4b.

For DES determination, isocratic elution with a low-potential electrochemical detector (detector 2, 0.30 V) was used. Frischkorn *et al.* [24] previously determined traces of oestrogenic growth-promoting hormones by HPLC with voltammetric detection by exploiting their different oxidation potentials. The use of a low oxidation potential allows greater

specificity to be achieved. Chromatograms relating to the standard DES solution and to an extract of beef muscle to which the latter had been added are shown in Fig. 5.

For the determination of oestradiol, its fluorescence emission was exploited. Oestradiol was eluted with the same fraction containing zeranol/zearalenone and their respective metabolites. Under the conditions of fluorescence detection, only oestradiol displays emission, which means that its determination is unaffected by other anabolic steroids. The chromatograms relating to the standard solution of oestradiol and an extract of bovine muscle spiked with this substance are shown in Fig. 6.

Evaporation

In all the methods in which samples are evaporated or concentrated in air or a nitrogen atmosphere, with or without heating, special care must be taken to avoid loss or structural modifications of the analytes. The significantly lower yields in the concentrated extracts are presumably due to partial adsorption of the anabolic steroid on the glass walls during evaporation of the extracts. This effect is observed in particular with DES, as its complete loss was observed during solvent removal. In order to reduce this problem and to obtain quantitative yields, silanized conical tubes were used in all operations involving concentration steps.

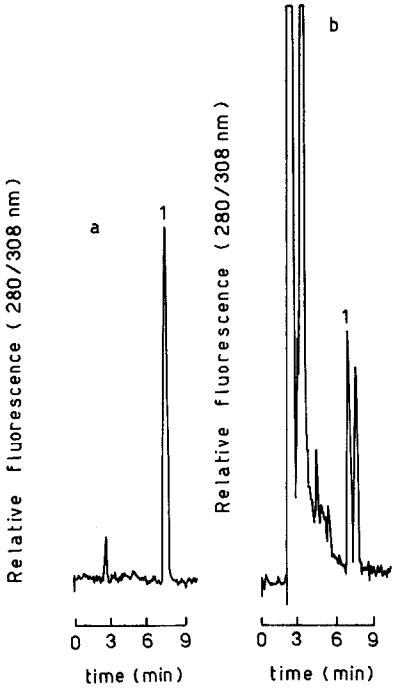


Fig. 6. HPLC of oestradiol with isocratic elution and fluorescence detection. Mobile phase, 0.01 *M* KH_2PO_4 (adjusted to pH 3.0 with H_3PO_4)–acetonitrile (54:46, v/v); flow-rate, 1.2 ml min^{-1}; fluorescence detection, λ_{ex} = 280 nm, λ_{em} = 308 nm. (a) Standard solution: 1 = oestradiol. (b) Chromatogram of extracted beef muscle spiked with (1) oestradiol (9 ng/g).

Solvent flow-rate

The solvent flow-rate through the column system was found to affect the recovery of anabolics from tissue extracts. With the Carbopack B column percolation flow-rates between 1 and 5 ml min^{-1} did not affect the recovery as Carbopack B has a surface area of 100 m^2 g^{-1} and interactions between the surface and analyte occur instantaneously.

The flow-rate through the resin column is more critical. Losses of zeranol/zearalenone and their respective metabolites, DES and oestradiol were observed when dichloromethane–methanol (70:30, v/v) was passed through the column at flow-rates higher than 1 ml min^{-1}.

Reusability

The reusability of both the Carbopack B and Amberlite columns was evaluated by performing repeated extractions of anabolics from homogenate tissue samples. After each use cycle the Carbopack B column was regenerated with 5 ml of dichloromethane followed by 5 ml of methanol. Resin regeneration was achieved using 20 ml of 0.05 *M* sodium hydroxide solution followed by 1 ml of water.

It was found that the entire solid-phase extraction system could be used only twice because, during the third cycle with homogenate tissue, the Carbopack B column became partially inefficient. This was probably due to the chemioadsorption of proteins on the carbon surface.

Plastic cartridges

As columns containing phases for solid-phase extraction have in recent years been made of plastic, experiments were carried out to see whether the cartridges could be packed with the phases used here. During the development of procedures to carry

out these analyses with plastic cartridges, the existence of a number of extraneous peaks was observed. These peaks varied according to the solvent used to elute the cartridges. A larger number of interferences were noted when mixtures containing dichloromethane were used. These interferents are present in the polypropylene of the cartridge and in the polyethylene of the frits. Repeated Soxhlet extraction with ethyl acetate and dichloromethane did not solve the problem of removing these interferences due to the commercial cartridges. For these reasons, plastic cartridges cannot be used, so that in the method proposed here, glass columns must be employed.

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