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DETECTION OF ESTROGEN-LIKE COMPOUNDS BY THIN-LAYER CHROMATOGRAPHY

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SUMMARY

There are different national food regulations concerning the use of hormone-active substances in the breeding of domestic animals that serve as human food. This situation requires specific and sensitive methods for the detection of any residues in food. The hormones used in practice can be determined with improved sensitivity by coupling to the diazonium compound Fast Dark Blue R Salt. After thin-layer chromatography on high-performance thin-layer chromatographic plates with two different solvent mixtures, zeranol, estradiol, estriol and estrone are analysed as azo dyestuffs. The detection limit of these hormones is below 10 ng.

INTRODUCTION

The use of hormone-active substances in animal breeding for improved meat and fat gain has become popular in some countries. In the U.S.A., hormone-like compounds, first applied in the production of poultry, have been used commercially in cattle breeding since 1954[1]. The application is approved, provided that the meat and organs of the slaughtered animals are free from any residues.

The food regulations in Germany do not allow the application of any hormone-active substances for growth promotion and fattening of cattle, sheep, swine, goats, rabbits, poultry or fish[2,3]. Nevertheless, estrogenic compounds are approved for medical purposes under the control of a veterinarian. The same regulations are valid for the EEC countries and other European countries, *e.g.*, Austria[4–6].

The Thai government has not issued any regulations in this respect. However, imported meat products, especially poultry, which contribute considerably to Thailand's animal food supply, may contain estrogen residues as a result of different legal requirements in the exporting countries. Therefore, the Bangkok Ministry of Health is also interested in suitable methods for the detection of estrogen residues in meat products.

Formerly, the synthetic hormones diethylstilbestrol and the stilbene derivatives hexestrol and dienestrol were the most widely used substances, applied as implants or additives to feed. Today, the natural steroidal estrogen estradiol and its esters, or zeranol, a derivative of the mycoestrogen zearalenone, are in common use. These substances are sometimes applied in combination with progesterone or testosterone, usually as implants or injections and rarely as additives to feeds[7].

The low concentrations of residues expected in animal tissues after illegal application of hormone-active substances require very sensitive detection methods[8]. In recent years, several investigations on the residue analysis of hormone-like compounds in meat have been published. The biological test is very sensitive to estrogenic activity, but does not permit the differentiation of single compounds[9]. Therefore, specific and sensitive chemical methods are important. Thin-layer and gas-chromatographic procedures have been proposed by several workers[1,10–12]. The most sensitive and specific determination of estrogens may be performed by modern radioimmunoassay, but this technique requires special biochemical reagents and expensive laboratory equipment[13]. Thin-layer methods are very suitable as screening tests in the analysis of hormone residues because of their modest instrumental and time requirements[12]. Recently, a quantitative colorimetric analysis of steroidal estrogens, coupled with a diazonium compound (Fast Dar[®] Blue R Salt) and separated by thin-layer chromatography (TLC), has been developed in clinical chemistry[14]. This method has also been proposed for the analysis of hormone residues in meat[15]. A similar combined TLC–spectrophotometric method is suitable for the detection of zeranol in veal[16].

As a further development of existing methods, we have worked out a quantitative colorimetric determination of zeranol and estradiol in the presence of estriol and estrone. The latter two compounds may be expected in animal tissues as metabolites of estradiol.

EXPERIMENTAL

Apparatus

The apparatus used consisted of a homogenizer (Moulinette), a mechanical shaker, centrifuge tubes (50 ml), a chromatographic column (600 × 25 mm), chromatographic tanks, a rotary vacuum evaporator, a waterbath, a microlitre syringe and a densitometer (Chromatogramm-Spektralphotometer PM 3; Zeiss, Oberkochen, G.F.R.).

Reagents and reference compounds

Silica gel 60 HPTLC plates and Fractogel 6000 PVA were used.

Tetrahydrofuran, ethanol, chloroform, benzene, acetonitrile, dichloromethane, cyclohexane, diethyl ether, ethyl acetate, sodium hydrogen carbonate and sodium carbonate were of p.a. grade or residue-analysis quality (all obtained from Merck, Darmstadt, G.F.R.). β-Glucuronidase/arylsulphatase from *Helix pomatia* was purchased from Boehringer (Mannheim, G.F.R.), estradiol, estrone and estriol from Serva (Heidelberg, G.F.R.) and zeranol from TAD Pharmazeutisches Werk (Cuxhaven, G.F.R.). Fast Dark Blue R Salt (2,6-dichloro-4-nitro-1-diazo-2,5-dimethoxybenzene-4-diazonium chloride) was a product of Searle Diagnostic (London, Great Britain).

Stock solutions

The hormones were dissolved in ethanol (1 mg/ml). The aqueous solution of Fast Dark Blue R Salt (10 mg/ml) was prepared freshly. To sample of 100 μg of each reference substance, 1 ml of the dyestuff solution was added, followed by 0.5 ml of a saturated solution of sodium hydrogen carbonate. After a reaction time of 10 min, the azo dye of each estrogen was extracted with benzene, the azo dyes of zeranol and estriol being extracted with 10 ml and the reaction products of estradiol and estrone with 5 and 2 ml, respectively. The separated benzene solutions, filtered through anhydrous sodium sulphate, served as standard solutions for calibration measurements.

Thin-layer chromatography

Aliquots of the samples were spotted with a microlitre syringe on HPTLC plates on a starting-line 2 cm from the edge. The plate was developed twice with diethyl ether–cyclohexane (80:20) (solvent mixture I). For the separation of zeranol and estriol, chloroform–ethanol (95:5) was used as the solvent (solvent mixture II). To separate all four azo dyes, development in solvent I was followed by redevelopment in solvent II.

Quantitative determination

After evaporation of the solvents, the plates were subjected to densitometric measurement of the separated red estrogen azo dyes. Optimal instrumental conditions were as follows: wavelength, 520 nm; slit, 3.5 × 6 mm; slit-width of the monochromator, 0.05 mm; distance between plate and instrument head, 2 mm; sensitivity, 200 mV; and scanning and chart speeds, 120 nm/min. The densitometer was adjusted to 100% with the colourless background. The plate travelled in the direction of chromatography. The peak areas on the chart were calculated by triangulation.

Calibration

For calibration of the thin-layer chromatographic and densitometric methods, we used solutions of the estrogen azo dyes, diluted 1:5 with benzene. The calibration range was from 2 to 40 ng for zeranol and estriol, from 4 to 80 ng for estradiol and from 10 to 200 ng for estrone.

Extraction and clean-up

For preliminary experiments on the extraction of added hormones from meat and liver, we applied methods described elsewhere[6,15]. A 50-g amount of meat was minced, mixed with 100 ml of ethanol and extracted by shaking for 1 h. After centrifugation at 2500 *g*, the supernatant was decanted and the extraction procedure was repeated twice by stirring the residue with 100 ml of ethanol.

The combined ethanolic extracts were evaporated on a rotary vacuum evaporator at 40°. The residue was treated with 10 ml of dichloromethane and, after addition of 10 ml of water, the organic phase was separated. Two further extractions with 20-ml portions of dichloromethane were carried out. For complete extraction of free and conjugated steroids from tissues, an enzymatic treatment of the aqueous fraction with β-glucuronidase/arylsulphatase, followed by solvolysis according to proposed methods[15,16], should be added. The combined dichloromethane extracts

were washed three times with 10 ml of 10% sodium carbonate solution (pH 10.5) and evaporated under vacuum. The residue, dissolved in 5 ml of tetrahydrofuran, was separated from lipids by gel chromatography on Fractogel 6000 PVA with tetrahydrofuran, according to Günther[15]. The fraction eluted with 125–250 ml, containing the steroids and zeranol, was collected and concentrated under vacuum. The residue was dissolved in 30 ml of benzene and washed twice with 10 ml of saturated sodium chloride solution. Steroidal estrogens and zeranol were extracted from benzene solution with 30 ml of 1% sodium hydroxide solution. The aqueous phase, collected with 20 ml of water in a separating funnel, was acidified with 2 ml of concentrated hydrochloric acid and re-extracted with benzene (3 × 15 ml). After washing the benzene phase with water to neutrality, it was dried with anhydrous sodium sulphate and evaporated to dryness on a rotary evaporator. The residue was dissolved in 1 ml of ethanol, re-evaporated and the final residue dissolved in 0.1 ml of ethanol.

By adding 1 ml of a solution of Fast Dark Blue R Salt and 0.5 ml of sodium hydrogen carbonate solution, as described for standard compounds, we coupled the extracted compounds to the diazonium compound. The reaction products were extracted with 5 ml of benzene and used for the quantitative determination. An aliquot of reference sample, spotted on the same plate, served for the identification and quantitative determination of the steroidal estrogens and zeranol.

RESULTS AND DISCUSSION

The coupling of steroidal estrogens with Fast Dark Blue R Salt has been found to be a sensitive and specific detection method for these hormones[14,15]. The reaction of zeranol, a resorcylic acid μ-lactone, with Fast Dark Blue R Salt has not been described before. Under the experimental conditions, zeranol formed the most intense azo dye of the compounds examined. The absorption maximum of the blue-violet compound was at 550 nm, while the red or red-violet derivatives of the steroidal estrogens showed absorption maxima between 520 and 490 nm (Table I). At 520 nm, all compounds showed sufficient absorption for quantitative determination.

In thin-layer chromatography on Silica gel 60 HPTLC plates, the solvent mixture chloroform–ethanol (95:5) proved optimal for the separation of zeranol and estriol, but did not separate zeranol and estradiol. Moreover, in this solvent system, the spot of the estrone dye was superimposed on two interfering spots, resulting from extracted Fast Dark Blue R Salt. In diethyl ether–cyclohexane (80:20), estradiol and estrone were sufficiently separated, but zeranol and estriol remained near the starting line. Development with this solvent mixture, followed by a second development with

TABLE I

ABSORPTION MAXIMA OF ESTROGENS COUPLED WITH FAST DARK BLUE R SALT

Compound	$\lambda_{max.}$ *in benzene (nm)*
Zeranol	550
Estriol	520
Estradiol	514
Estrone	490 (425)*
Fast Dark Blue R Salt	326, 425

* Supposed to result from extracted Fast Dark Blue R Salt.

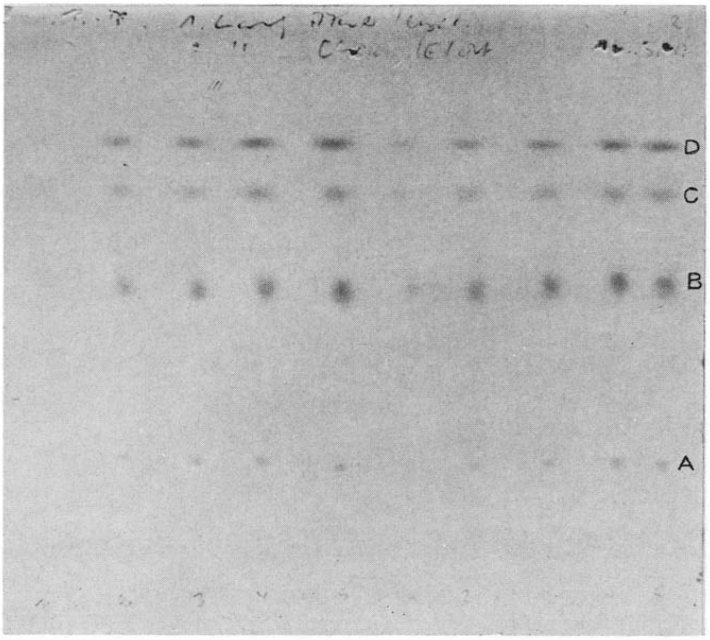

Fig. 1. Chromatogram of standard mixtures on silica gel 60 HPTLC plates, developed, in succession, with diethyl ether–cyclohexane (80:20) and chloroform–ethanol (95:5). A = Estriol; B = zeranol; C = estradiol; D = estrone.

chloroform–ethanol, resulted in sufficient separation of all four compounds for quantitative determination (Fig. 1).

The calibrations were performed with the benzene solution of each estrogen and also with their mixture, coupled with Fast Dark Blue R Salt. The benzene standard solutions proved to be stable for over 6 months when stored in a refrigerator.

The data in Fig. 2 resulted from different determinations of zeranol and

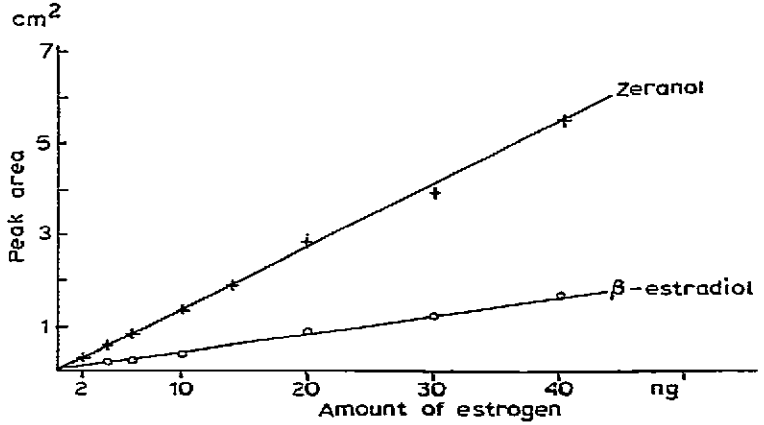

Fig. 2. Calibration graph for quantitative densitometric determination of zeranol and estradiol coupled with fast Dark Blue R Salt and separated by TLC.

estradiol at concentrations from 2 to 40 ng per spot. The different slopes of the calibration graphs illustrate the high sensitivity of the zeranol detection. For accurate results, it is necessary to run standard solutions along with the samples.

In recovery experiments, we extracted 40 μg of zeranol and estradiol added to 50 g of meat. Gel permeation chromatography followed by solvent partition resulted in the purest extracts. On the basis of the results of our previous experiments, the recovery of the four estrogens, determined by the described procedure, was about 50%. Residues of zeranol might be detected down to concentrations of 1 μg/kg and estradiol, estriol and estrone down to less than 10 μg/kg in meat.

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