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APPLICATION OF SORBENT EXTRACTION CHROMATOGRAPHY TO THE PURIFICATION OF DIETHYLSTILBOESTROL EXTRACTED FROM MUSCLE TISSUE AND DETERMINED BY RADIOIMMUNOASSAY

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SUMMARY

A chromatographic procedure is described for the purification of bovine muscle tissue extracts prior to the determination of diethylstilboestrol (DES) by radioimmunoassay. Sorbent extraction chromatography of tissue extracts on reversed-phase octadecyl (C_{18}) columns gives adequate recovery of residue and a suitable sample for radioimmunoassay. This procedure, which is simple and rapid, provides an alternative to a more complex purification by high-performance liquid chromatography. Using this method, the limit of detection for DES in muscle samples is approximately 40 pg/g.

INTRODUCTION

Extensive monitoring for residues of illegally used anabolic agents in animal production is required by European Community (EEC) regulations [1]. Rapid, sensitive and accurate techniques are needed and radioimmunoassay (RIA) has been proposed as an EEC reference method for stilbene residues [2].

Tissue sample preparation methods based on solvent extraction and liquid–liquid partitioning do not give a sufficiently purified sample extract for accurate quantitation by RIA of diethylstilboestrol (DES) [3]. To overcome this problem methods for DES in tissues include a final purification of the extract by column chromatography on Sephadex LH-20 [3], by high-performance liquid chromatography (HPLC) [2], or by a combination of these two techniques [4] prior to the RIA.

In the case of measurement of DES in urine samples, there can be a major problem with false positive results in the RIA unless a chromatographic step is introduced [5]. The use of a reversed-phase octadecyl (Bond Elut C_{18}) column has been described as an extraction and purification technique for DES in urine [6].

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This paper describes the combining of the solvent extraction and liquid–liquid partitioning clean-up procedure of the EEC method for DES in muscle with the use of reversed-phase octadecyl (Bond Elut C_{18}) column chromatography to provide suitably purified muscle extracts for determination of DES by RIA.

EXPERIMENTAL

Chemicals

Diethyl ether (pro anaesthesia) from May & Baker (Dagenham, U.K.), hexane (pesticide grade) from Fisons (Loughborough, U.K.), methanol and toluene (Aristar grade) and *n*-butanol and ethyl acetate (Analar grade) from BDH (Poole, U.K.), acetonitrile (HPLC grade) from Rathburn Chemicals (Walkerburn, U.K.) and double-distilled water were used. Other chemicals were Analar grade from BDH. Sephadex LH-20 from Sigma (St. Louis, MO, U.S.A.) and reversed-phase octadecyl Bond Elut C_{18} (100 mg) columns from Analytichem International (Harbor City, CA, U.S.A.) were used for the chromatographic step.

DES from Sigma was used for fortification of muscle samples and as standards in the RIA. [^{3}H]DES from Amersham (Bucks., U.K.) with a specific activity of 3.31 TBq/mmol was used for estimating recovery and for the RIA. The DES antiserum (Laboratoire d'Hormonologie, Marloie, Belgium) used in the RIA had principal cross-reactivity (relative to 100% for DES) of 9.7% for *trans*-DES di-*n*-propionate, 3.3% for hexoestrol, 1.0% for dienoestrol and 0.4% for *trans*-DES dimethyl ether.

Samples

Samples of neck muscle were taken from heifers and steers at slaughter and stored at −20°C prior to analysis. The animals selected were from a production trial designed to evaluate the growth response to implantation with the anabolic agents oestradiol and trenbolone acetate. None of the animals had been treated with DES but one half of each type had been implanted with oestradiol and trenbolone acetate and the others had received no anabolic agents. Samples from non-implanted and implanted animals were included in this study to assess the performance of the assay technique for DES both on samples from animals not implanted with any anabolic agents and on samples from animals implanted with anabolic agents other than DES.

The method was validated by analysis of fortified samples. Levels of 150, 750 and 1500 pg DES, in 0.1 ml methanol, were added to muscle tissue samples (1.5 g) prior to extraction.

Extraction and clean-up

The extraction and clean-up procedure was according to the draft reference method for stilbenes of the EEC working group (muscle, method 2) but omitting the HPLC step [2]. This method involves preliminary extraction of the sample (1.5 g) with diethyl ether, liquid–liquid partitioning between sodium hydroxide (1 *M*) and diethyl ether–*n*-butanol (9:1), acidifying with hydrochloric acid (1 *M*) and extraction with diethyl ether.

Column chromatography

The extracts were further purified by column chromatography on Sephadex LH-20 columns or on reversed-phase octadecyl Bond Elut C_{18} columns.

Sephadex LH-20 was swollen overnight in the eluent (toluene–methanol, 90:10) and slurried into a glass column (0.5 cm I.D.) to a height of 9 cm. The sample extract was evaporated to dryness, redissolved in 0.2 ml of eluent and placed on top of the column. The column was eluted with 11 ml of eluent. The first 6 ml were discarded and the further 5 ml collected, evaporated to dryness and redissolved in 1.5 ml methanol. The columns were regenerated by addition of a further 30 ml of eluent.

Chromatography on reversed-phase C_{18} columns was according to the method of Gaspar and Maghuin-Rogister [6] for DES in urine. The sample extract was evaporated to dryness, redissolved in 0.2 ml methanol and then diluted to 5 ml with water. After absorption of DES on to the column, washing successively with 1 ml of methanol–water (55:45) and 1 ml of hexane and drying under vacuum, the column was eluted with 1 ml of ethyl acetate. The collected fraction was evaporated to dryness and redissolved in 1.5 ml of methanol. The columns were regenerated by washing with 2 ml of methanol and drying under vacuum.

The separation of *cis*- and *trans*-isomers of DES by the two chromatographic columns was determined using HPLC. Since *cis*-DES is not commercially available, a methanolic solution of the DES standard, with an apparent content of 11% *cis*-DES, was used. DES (10 μg) was chromatographed on the Sephadex LH-20 and Bond Elut C_{18} columns and the fractions from these columns were analysed by HPLC. The chromatographic analysis was performed using the following equipment: Model 6000A solvent delivery system, Z-module radial compression separation system and Model 440 UV detector (254 nm) (Waters Assoc., Milford, MA, U.S.A.). The separation was performed on a Novapak C_{18} (4 μm) Radial-Pak cartridge (Waters Assoc.) with a mobile phase of acetonitrile–water (60:40) at 2 ml/min. Under these conditions, the retention times were 3.5 and 5.7 min for *trans*-DES and *cis*-DES, respectively.

Radioimmunoassay

Recovery of [^{3}H]DES, added to the sample prior to extraction and clean-up, was estimated on 0.3 ml of the purified extract. RIA, on 0.6 ml of the purified extract, was according to the procedure outlined in the EEC working group method [2]. The tissue extracts and standards, after evaporation of methanol, were reconstituted in 0.06 ml of methanol (to ensure that DES is dissolved) and then 0.54 ml of gelatin–phosphate buffer was added.

RESULTS AND DISCUSSION

The isomerisation of DES can influence the quantitation of residue in samples [5, 7]. A suitable sample extraction and purification procedure should recover both isomers or selectively recover the *trans*-DES isomer, which is the biologically active form of DES. On the Sephadex LH-20 column, under conditions optimised for *trans*-DES recovery, there was only partial separation of *cis*-DES with

about 50% being recovered in the 5-ml fraction collected for RIA. Approximately 90% of the *trans*-DES was recovered in this fraction. On the Bond Elut C_{18} column both isomers were substantially recovered — approximately 90% of the *cis*-DES and 80% of the *trans*-DES — in the ethyl acetate fraction collected for RIA.

Recovery of [^{3}H]DES, added to the muscle samples prior to extraction, was similar for column chromatography by either Sephadex LH-20 or Bond Elut C_{18}. Typical mean recovery for the extraction and clean-up procedure involving Bond Elut C_{18} chromatography is 53.9% (S.D. = 7.2%, n = 26). Both chromatographic systems provided sample extracts suitable for quantitation by RIA, but the Bond Elut C_{18} system is preferred because of its greater simplicity and rapidity.

Using Bond Elut C_{18} chromatography, parallelism and near-coincidence is achieved for standard curves prepared with and without extract (Fig. 1A). This contrasts with the extreme non-parallelism found where the final purification on Bond Elut C_{18} was omitted (Fig. 1B).

Results for the analysis of muscle samples fortified with various levels of DES prior to extraction are shown in Table I. A linear regression between the amount of DES (100–1000 pg/g) added to the muscle samples and the concentration determined by RIA has a correlation coefficient of 0.99. The apparent levels of DES in muscle samples from untreated animals are shown in Table II. No differences in apparent levels of DES were found between animals implanted or not implanted with the anabolic agents oestradiol and trenbolone acetate. The mean and S.D. values allow for calculation of the limit of detection at approximately 40 pg/g (mean + 2 S.D.).

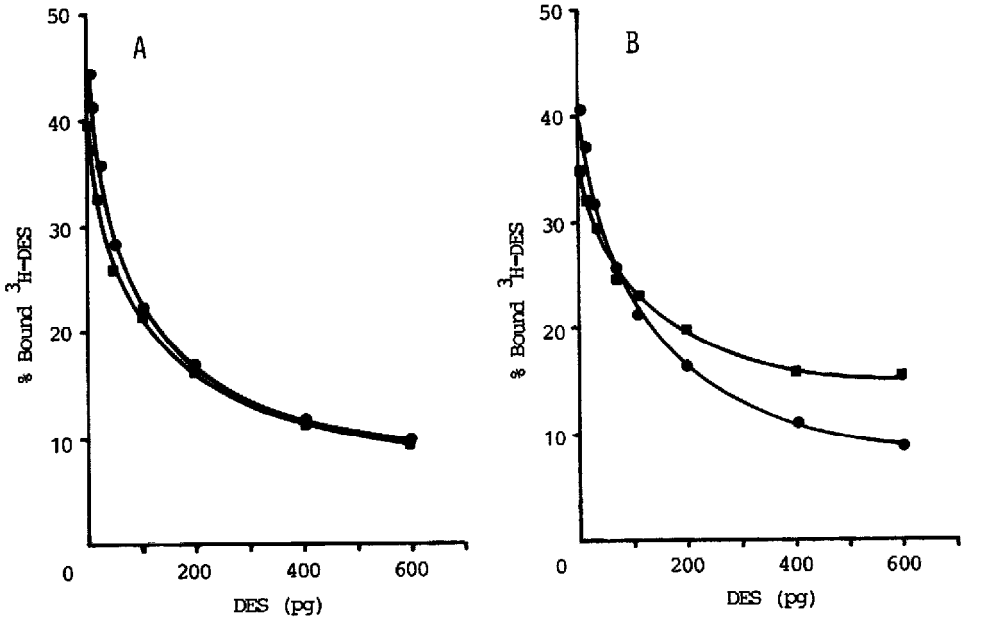

Fig. 1. Standard calibration curves for DES, with and without muscle extract. (●) Standards without extract; (■) standards with extract. (A) Extract prepared using reversed-phase octadecyl C_{18} column chromatography. (B) Extract prepared without any column chromatography.

TABLE I

DETERMINATION OF DES IN FORTIFIED MUSCLE SAMPLES BY RADIOIMMUNOASSAY

DES added (pg/g)	*n*	DES determined (mean ± S.D.) (pg/g)	C.V. (%)
0	6	6± 6	100
100	4	93±15	16
500	4	464±62	14
1000	6	1080±74	7

TABLE II

APPARENT DES LEVEL IN MUSCLE SAMPLES FROM UNTREATED ANIMALS DETERMINED BY RADIOIMMUNOASSAY

Animals	Treatment[a]	*n*	DES determined (mean ± S.D.) (pg/g)
Steers	Not implanted	8	6± 9
	Implanted	8	7± 7
Heifers	Not implanted	8	10±13
	Implanted	7	12±15

[a]Animals not implanted or implanted with the anabolic agents oestradiol and trenbolone acetate.

Introduction of this chromatographic step at the end of the extraction and clean-up procedure in the draft EEC reference method provides a simple, inexpensive and reliable screening method for DES in muscle samples. The Bond Elut C_{18} columns were reused for up to ten samples without there being any detectable reduction in their capacity to purify the sample extract and maintain the recovery of [^{3}H]DES. All the washing steps applied to urine samples [6], involving methanol–water (55:45) and hexane, may not be necessary with muscle because of the preliminary clean-up of the muscle extract by liquid–liquid partitioning. The technique is much more rapid and requires less expensive equipment and costs than HPLC and is suitable unless specific separation of the different stilbenes is required.

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