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Note

Rapid extraction and purification of diethylstilboestrol in bovine urine hydrolysates using reversed-phase C_{18} columns before determination by radioimmunoassay

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Several methods can be used for the determination of diethylstilboestrol in urine samples collected from slaughtered cattle. Radioimmunoassay allows the analysis of large numbers of samples and is thus well suited to the screening of animals illegally treated with stilbenic estrogens. Its main limitation for this purpose is often a bad specificity of the antibodies, which results in false positive results, *e.g.* it is necessary to extract and purify diethylstilboestrol residues from urine before their analysis by radioimmunoassay. These extraction and purification procedures are time-consuming, they increase the cost of the analysis and they limit the number of samples that can be analyzed per day.

In this paper, we describe a chromatographic procedure using a reversed-phase octadecylsilane (C_{18}) for the extraction and purification of diethylstilboestrol from urine hydrolysates before its determination by radioimmunoassay. It avoids the troublesome extraction by diethyl ether and is performed with low-cost equipment. It gives reliable, reproducible results and, owing to its simplicity and rapidity, the cost of the analysis was lowered and the efficiency of the laboratory in charge of diethylstilboestrol control in bovine urine samples was increased.

MATERIALS AND METHODS

Reagents and solvents

Reagents used for the radioimmunoassay of diethylstilboestrol have been described previously[1]. Analytical grade (Merck) ethyl acetate, hexane and methanol were distilled in an all-glass apparatus.

Apparatus

A "Vac Elut Vacuum" box with a capacity of ten columns was used. It was connected to a water vacuum pump equipped with a manometer. This apparatus and disposable reversed-phase octadecyl (Bond Elut C_{18}) (100 mg) columns were from Analytichem International, Harbor City, CA, U.S.A.

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Column treatment

The steps of conditioning, washing and elution of the columns were performed under a pressure of 400 mmHg. Columns were washed before use with successively 2 ml of methanol and 2 ml of distilled water. For this operation, the columns must not be dried.

Sample urine

One set of 39 urine samples was prepared by dilution of urine, rich in diethylstilboestrol residues, from treated animals using various volumes of urine from untreated cattle. The diethylstilboestrol concentration of the starting urine (rich in diethylstiboestrol residues) had been determined in a cooperative study in the Netherlands between four laboratories using radioimmunoassay with various chromatographic clean-up procedures, including high-performance liquid chromatography, and two different antibodies.

Another set of 59 urine samples was prepared using urine from untreated cattle and pure diethylstilboestrol in known amounts.

Urine hydrolysis and purification

For the hydrolysis of glucurono- and sulpho-conjugates of diethylstilboestrol, 200 μl of urine were mixed with 300 μl of hydrolysis buffer (15 ml of 0.02 *M* phosphate buffer (pH 7) containing 500 μl of *Helix pomatia* suc (Boehringer) and 5000 cpm of tritiated diethylstilboestrol (Amersham)). The samples were incubated at 37°C for 1 h. The hydrolysates were then diluted with 5 ml of distilled water, layered at the top of the columns and adsorbed under vacuum until the level of the solution reached the surface of solid phase. The columns were washed successively with 1 ml of methanol–water (55:45) and 1 ml of hexane. At this stage, the columns were left under suction for 5 min. Adsorbed diethylstilboestrol was eluted with 500 μl of ethyl acetate and collected in disposable glass tubes. Solvent was then evaporated to dryness and 500 μl of ethyl acetate were added again to dissolve the diethylstilboestrol. Then 300 μl of the eluate were evaporated to dryness and used for the radioimmunoassay, while the radioactivity of 100 μl portions was determined in order to calculate the recoveries.

Radioimmunoassay

Dried residues from the 300 μl of eluate were solubilized by the successive addition of 300 μl of phosphate–gelatin buffer (0.02 *M* phosphate buffer, pH 7.4, containing 0.05% of gelatin), 100 μl (10 000 cpm) of tritiated diethylstilboestrol solution in ethanol and 100 μl of the antibody dilution (1:20 000).

A standard curve was established using tubes containing increasing amounts of diethylstilboestrol (0, 6.25, 12.5, 25, 50, 100, 200, 400, and 800 pg) and 200 μl of phosphate–gelatin buffer, 100 μl of tritiated diethylstilboestrol and 100 μl of the antibody solution in the conditions described above for unknown samples.

The tubes were incubated first at 37°C for 30 min and then at 4°C for 2 h. Then 500 μl of charcoal–dextran suspension (5 g of Norit and 0.5 g of dextran per litre of phosphate–gelatin buffer) were added to each tube at 4°C and left at the same temperature for 15 min. After centrifugation at 1500 *g* for 10 min, 500 μl of the supernatant were taken and mixed with 4 ml of Beckman HP/B scintillation cocktail. The radioactivity was determined using a Beckman LS 1800 counter.

RESULTS AND DISCUSSION

Radioimmunoassay of diethylstilboestrol, after adsorption and purification on reversed-phase C_{18} columns, was performed on 22 urine samples from untreated animals and 76 samples of known diethylstilboestrol concentration at 14 different levels (Table I).

The chromatographic procedure on reversed-phase C_{18} columns described in this paper allowed the simultaneous purification of ten urine samples within 15 min. The official celite method, which involves ether extraction and purification on celite[3], takes 3 h.

Dilution of the urine hydrolysates with 5 ml of distilled water improved the adsorption of diethylstilboestrol on the column before its elution and led to a higher percentage recovery. When ethyl acetate was used as eluent, blank values (of urine from untreated animals) were *ca.* 30 pg/ml and the recoveries were 85%.

Nevertheless, in the case of urine highly contaminated with blood or feces, it is essential to wash the columns successively with methanol–water and hexane. This treatment lowered the interferences from the biological medium, but resulted in lower recoveries (*ca.* 55%).

In Table I, the rather high mean value for urines from untreated animals can be explained because 18 of the 22 samples were rather dark and contaminated with blood and feces. In another series of normally coloured samples, the mean value was 0.07 ppb. For sample containing diethylstilboestrol, the equation of the regression line between known residue concentrations (y) and values found by radioimmunoassay (x) was as follows: $y = 0.069 + 0.936x$, $r = 0.982$ ($F^1_{13} = 351$, $P<0.001$).

TABLE I

COMPARISON BETWEEN URINARY DIETHYLSTILBOESTROL RESIDUE CONCENTRATIONS DETERMINED BY RADIOIMMUNOASSAY AND KNOWN VALUES

Number of samples	*Known concentration (ppb)*	*Mean percentage recovery*	*Mean concentration determined by RIA (ppb)**	*Standard deviation*	*CV (%)*
22	0.00	55	0.26	0.14	55
8	0.30	53	0.29	0.07	25
2	0.40	52	0.41	0.04	9
2	0.50	53	0.39	0.06	15
10	0.60	58	0.69	0.19	27
3	0.70	53	0.57	0.18	31
4	0.80	52	0.62	0.17	28
13	0.90	59	0.97	0.31	32
3	1.00	53	0.77	0.09	12
2	1.10	50	1.00	0.42	42
9	1.20	54	1.30	0.34	25
1	1.40	53	1.32	–	–
2	1.50	53	1.38	0.04	3
9	1.80	57	2.00	0.57	28
8	3.00	60	3.16	0.41	13

* The American billion (10^9) is meant.

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