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Note

Identification of thyrostatic drug residues in animal thyroids by high-performance thin-layer chromatography and fluorescence reaction detection

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An interesting technique for the detection of sulphur-containing organic compounds on thin-layer plates has been described[1–3]. It is based on a ligand-exchange reaction between the palladium(II)–calcein complex and organo-sulphur compounds. The release of free calcein provides an indirect measurement of the amount of the organo-sulphur compounds through fluorescence detection.

Recently, this reagent was also indicated for post-column detection in liquid chromatography for selected classes of sulphur-containing compounds[4]. According to the authors, the fluorescence intensity of the reagent solution was enhanced by adding some zinc(II).

We have found this latter reagent to be useful in the detection, on thin-layer plates, of thyrostatic drugs such as methylthiouracil which may be present as a residue in the thyroid gland of cattle illegally treated with these prohibited drugs.

EXPERIMENTAL

Apparatus

The following apparatus was used: chromatographic tanks 27 cm × 12 cm × 21 cm; transilluminator, Blak-Ray C-62 (365 nm) obtained from Ultra-Violet Products, (U.S.A.).

Chemicals and reagents

Calcein was obtained from E. Merck (Darmstadt, F.R.G.). A stock solution (10^{-3} *M*) was prepared by dissolving 62.2 mg of the indicator in 3 ml of 0.1 *M* sodium hydroxide and diluting to 100 ml in water. The solution was stored at 4°C and was stable for at least 30 days.

Palladium(II) chloride was obtained from Carlo Erba (Milan, Italy). A stock solution ($5 \cdot 10^{-3}$ *M*) was prepared by dissolving 44.4 mg of the salt in 5 ml of 0.1 *M* hydrochloric acid and, after standing 24 h, diluting to 50 ml The solution was stable for at least 50 days. A stock solution of zinc sulphate ($1.25 \cdot 10^{-3}$ *M*) was prepared by dissolving 22.4 mg of the monohydrate salt in 100 ml of water.

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The following thyrostatics were used: 2-thiouracil (TU); 6-methyl-2-thiouracil (MTU); 6-propyl-2-thiouracil (PTU); 6-phenyl-2-thiouracil (PhTU) (Carlo Erba); 2-mercapto-1-methylimidazole (TAP) (Aldrich Europe).[4] Stock solutions of these compounds were prepared in methanol at a concentration of 0.2 mg/ml, except for PhTU having one-half this concentration. The working solutions were prepared, as needed, by dilution in the same solvent.

All other chemicals and solvents were reagent-grade quality.

Extraction and clean up

The extraction of bovine thyroid glands and the successive purification of the extracts by means of Sep-Pak silica cartridges were performed according to the procedure described by Moretti *et al.*[5]. The residue obtained in this manner was dissolved in 200 μl of methanol (sample extract).

Preparation of spray reagent

A 4-ml volume of calcein stock solution and 1 ml of palladium chloride stock solution were mixed and diluted to 25 ml in the phosphate buffer (0.067 *M*) pH 7.2. This solution was left to stand for 24 h and then 0.2 ml of zinc sulphate stock solution were added. This concentrated spray reagent was stable for at least 8 days. On the day of use, 5 ml of the concentrated spray reagent were mixed with 5 ml of the phosphate buffer solution (0.067 *M*) pH 7.2 and with 10 ml of acetone (working spray reagent).

Chromatography

Thin-layer plates (10 cm × 10 cm) coated with silica gel without a fluorescence indicator (Kieselgel 60, Merck) were employed. Samples were spotted on the plates with an Hamilton micro-syringe (No. 7110). The solvent systems used chloroform–acetone (70:30) (I) and ethyl acetate (II). Each chromatographic chamber was lined with filter-paper and was saturated with the appropriate solvent system for 2 h prior to use. Each glass tank was fitted with a smaller internal 100-ml capacity glass vessel in which 30 ml of the appropriate solvent system were placed just before the development.

One-dimensional development. A 10-μl volume of sample extract was spotted on a line, 0.5 cm wide, 1 cm from the bottom of the plate; 2 μl (20 ng) of the working solution of MTU were spotted separately in the same manner. The chromatography was performed with solvent I until the solvent front had moved 7 cm from the starting line. The plate was removed from the developing chamber and dried with the aid of cold air. It was then examined by the transilluminator (365 nm) and any UV fluorescent spots, eventually present, were marked on the plate with a pencil. Afterwards the plate was sprayed with the working spray reagent until the plate was wet. Then it was again examined by UV light (365 nm) and if a spot with yellow-green fluorescence with a R_F value equal to that of the reference MTU appeared in the sample, chromatography with the two-dimensional technique was performed on a second aliquot of sample extract.

Two-dimensional development. For sample application, 10 μl of sample extract were applied to the plate at point P (see Fig. 2). On the same plate, the movement of MTU in the two solvent systems was monitored by spotting 2 μl (20 ng) of the

reference solution of MTU on each of the lateral lines. After the first development with solvent I, when the solvent had moved 6 cm from the starting line, the plate was removed from the chamber and dried in cold air. The second development was then performed for 6 cm with solvent II. The plate was removed from the chamber, dried and examined under UV light before and after spraying as described previously. The identity of MTU in the sample was established by comparing the fluorescence colour and R_F values with those obtained for reference MTU.

RESULTS AND DISCUSSION

Reagent solution

Contrary to earlier observations made by Frei *et al.*[2] and Inglis and Nicholls[3], it was found that optimum fluorescence development was obtained by using a more dilute reagent together with thin-layer plates without a fluorescence indicator. The reagent composition was extensively studied, specifically with regard to the ratio calcein:palladium(II) and also to the amount of Zinc(II) to be added for the maximum fluorescence enhancement. The best composition of the spray reagent was $0.4 \cdot 10^{-4}$ *M* calcein; $0.5 \cdot 10^{-4}$ *M* palladium(II) and $25 \cdot 10^{-7}$ *M* Zinc(II).

Thin-layer chromatography (TLC)

Several solvent systems were studied to obtain the best separation of MTU from the biological matrix components present in the extracts. Along with MTU, we also studied the separation of other thyrostatic substances (TU, TAP, PTU and PhTU) to determine the possibilities for detecting them by the procedure proposed. As seen in Fig. 1, MTU, TU and TAP are well separated from the extract components. In system I, MTU shows an R_F value equal to that of TAP. In Fig. 2 the

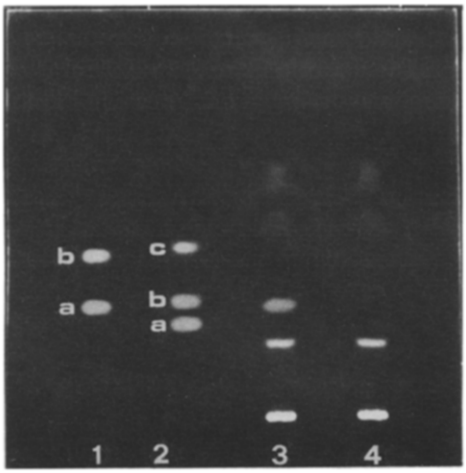

Fig. 1. One-dimensional TLC of bovine thyroid samples developed with solvent system I, then sprayed with the palladium(II)–calcein reagent and examined under UV light (365 nm). Lanes: 1 (a) MTU, 100 ng; (b) PTU, 100 ng; 2 (a) TU, 100 ng; (b) TAP, 100 ng; (c) PhTU, 100 ng; 3, thyroid extract sample contaminated with 1 ppm MTU; 4, thyroid blank extract.

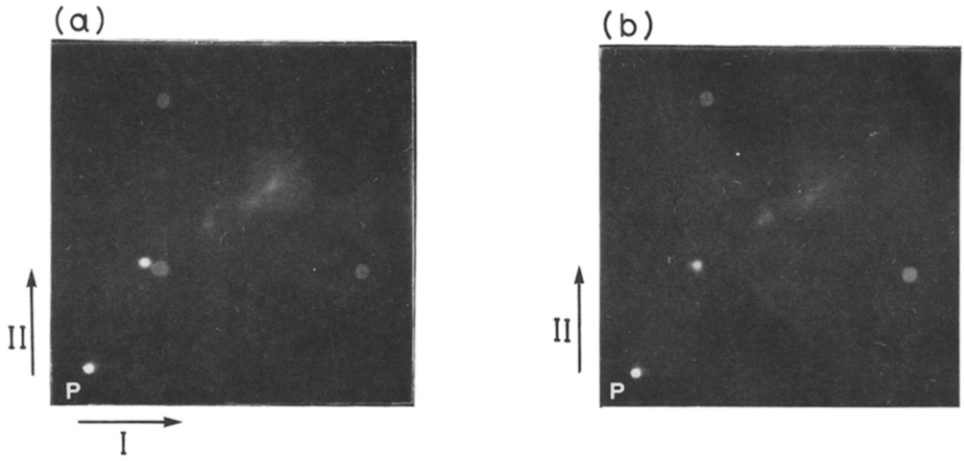

Fig. 2. Two-dimensional thin-layer chromatograms of bovine thyroid samples. (a) Thyroid extract contaminated with 2 ppm MTU (starting point P). References in side lanes: 100 ng MTU. Developing solvents I and II. Sprayed and viewed as in Fig. 1. (b) Thyroid blank extract (starting point P). Side lanes: MTU as in (a).

separation obtained for MTU with the two-dimensional technique is shown. In Fig. 1 and 2, a spot originating from the biological matrix is present near the MTU spot. This light blue fluorescent spot is present also before spraying and does not interfere with the identification of MTU. It should be noted that, in two-dimensional TLC, all five thyrostatics considered have distinct R_F values, as seen from Table I, where the R_F values obtained with the two solvent systems are listed.

Qualitative detection limit

Using the procedure described, the detection limit for MTU was 200 ppb (200 μg/kg). This limit is sufficient for tissue such as the thyroid because residues of this substance are very concentrated in this gland after an animal is treated illegally. For this reason, it may be necessary to use a more dilute solution of the sample extract in order to obtain well defined spots on thin-layer plates.

Application of the method

The method was applied to several samples of bovine thyroid glands taken at

TABLE I

TLC R_F VALUES OBTAINED WITH SOLVENTS I AND II

Thyrostatic	*Solvent I*	*Solvent II*
TU	0.28	0.60
MTU	0.34	0.61
TAP	0.35	0.43
PTU	0.47	0.83
PhTU	0.52	0.88

different slaughter-houses (about 50 samples). All samples giving positive results contained MTU residues. Those giving positive and negative results were analyzed in parallel by the method of Verbeke and De Brabander[6]. In this method, the thyrostatic substances are derivatized and detected as fluorescent spots after two-dimensional TLC and spraying with a cysteine reagent. The results obtained with the two methods were comparable.

CONCLUSIONS

The method proposed for detection of MTU is simple, does not require derivatization, is inexpensive and suitable for screening purposes. Regarding the other four thyrostatics, studies are in progress to find the best conditions for their separation from matrix components on thin-layer plates.

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