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METHOD OF ANALYSIS FOR DETECTING ANTI-THYROID SUBSTANCES IN FRESH MUSCLE TISSUE USING A SELECTIVE MERCURATED COLUMN

by

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WORKING DOCUMENT

Method of analysis for detecting anti-thyroid substances in fresh muscle tissue using a selective mercurated column.

1. Subject and scope :

Detection of anti-thyroid substances in fresh muscle tissue.

2. Principle :

Substances with thyreostatic action, also known as anti-hormones (AH), are extracted from tissues by means of methanol in presence of an internal standard. Percolation of the methanol extract through a mercurated adsorption column allows a selective and reproducible extraction of the AH.

After elution of the AH with an acid salt solution, the eluate is neutralized, buffered and allowed to react with NBD-Cl (7-chloro-4-nitrobenzo-2-oxa-1,3-diazole, e.g. Aldrich 16 326-0). This product reacts only with thiols and amines. After the reaction has taken place, the AH-NBD complexes formed are extracted in acid medium using diethylether. After it has been dried and evaporated down to the required volume, the diethylether fraction is put on to a thin layer plate, which is developped by the two-dimensional technique.

Before it has been sprayed, this plate is inspected for the presence of spots fluorescing at 366 nm. It is then sprayed with an alkaline cysteine or a cysteamine solution. By this means, the AH-NBD complexes which are not fluorescent are converted into cysteine-NBD complexes which are strongly fluorescent (limit of detection < 1 ng).

$$\text{NBD-Cl} + \text{AH.H} \longrightarrow \underset{\text{adsorbent combination}}{\text{AH-NBD}} + \text{HCl}$$

$$\text{AH-NBD} + \text{CYS.H.} \longrightarrow \underset{\text{fluorescent combination.}}{\text{CYS-NBD}} + \text{AH}$$

The AH are therefore detected in the form of spots which do not become fluorescent until after spraying (this is typical for these SH complexes). The identity of the anti-hormones is established by comparison of the Rf with reference substances.

With the procedure proposed, a minimum lower limit of detection for anti-thyroid substances (except phenylthiouracil and tapazole) of 25 ppb should be obtained using the bidimensional technique. In contrast to the other AH, the recovery of phenylthiouracil and tapazole from the mercurated column is 4 times lower so that for these substances the minimum lower limit amounts to 100 ppb.

The analyst should familiarise himself with the method and check that he can detect 25 µg/kg anti-thyroid substances in a spiked sample of muscle tissue.

3. Reagents

All reagents must be of analytical grade (p.a.).

3.1. NaOH 50% : dissolve 500 g NaOH in 500 ml water in a pyrex erlemeyer. Stopper and cool the solution to room temperature.

3.2. 5 M NaOH solution : dissolve 200 g NaOH in 1000 ml water

3.3. 4 M NaOH solution : dissolve 16 g NaOH in 100 ml water

3.4. acetic acid 0.5 M : dilute 28 ml glacial acetic acid (100%) to 1 l with water

3.5. acid salt solution : dissolve 29.2 g NaCl in 1 l 0.1 N HCl

3.6. 2,7-dibromo-4-hydroxymercurifluorescein solution (DBMF) : dissolve 500 mg DBMF in 200 ml water

3.7. HCl 7.5 M : dilute 63 ml HCl fumans (min. 37% HCl) with water to 100 ml

3.8. peroxide-free diethylether (e.g. anesthetic ether from Gifrer and Barbezat, Fr.)

3.9. Britton-Robinson buffer 0.4 M pH:8.0 : dissolve 24.73 g H_3BoO_3 in 800 ml hot water (60°C), cool and mix with 26.7 ml H_3PO_4 (85%), 23 ml glacial acetic acid (100%) and 68 ml NaOH 50%. Cool, check the pH by means of a pH-meter and adjust eventually. Dilute the solution to 1 l with water.

3.10. NBD-Cl solution : dissolve 5 mg NBD-Cl (7-chloro-4-nitrobenzo-2-oxa-1,3 diazole, e.g. Aldrich 16 326-0) in 1 ml methanol. Prepare this solution freshly each day and keep it in a cool dark place (see Section 5.11).

3.11. Preparation of the mercurated resin :
Dowex 1 x 2 (50-100 mesh) is first treated as follows : 20 ml of settled resin is suspended in water, transferred to a Buchner funnel (diameter 4 cm) and successively washed with 200 ml distilled water, 200 ml 0.5 M NaOH (e.g. 1/10 dilution of 5 M NaOH solution 3.2.), 200 ml distilled water, 200 ml 0.5 M acetic acid (3.4) and finally with 200 ml distilled water.
The resin is then transferred to a round bottomed flask (500 ml), mixed with 200 ml of an aqueous DBMF-solution (3.6) and rotated during 24 hours (e.g. on a rotatory evaporator without suction). The mercurated resin is then transferred to a Buchner funnel and washed with water until the eluate is colourless. The resin is then treated succesively with 200 ml of an acid salt solution (3.5.), 1 l distilled water, 200 ml 0.1 N NaOH (i.e. obtained by a 1/50 dilution of 3.2.) and finally with distilled water to neutral reaction (1 l). The mercurated resin is stored in a brown bottle in the dark.

3.12. thin layer silicagel 60 HPTLC plates without fluorescence indicator (e.g. Merck n° 5631 or 5547) (see section 6.1.)

3.13. spray solutions :
- solution I : mix 50 ml denaturated alcohol with 50 ml isopropanol. Add 2 ml 25% ammonia.
- solution II : dissolve 0.6 g cysteine hydrochloride (or 2-mercaptoethylamine) in 20 ml water. Keep this solution in a refrigerator at about 5°C. Make this solution freshly up each day.
- spray reagent : each day immediately before use, mix 2 ml solution II with 100 ml solution I.

3.14. Reference substances :

The AH used as reference substances are listed in the table below. Using these reference substances, prepare stock solutions containing 20 mg AH per 100 ml methanol. For MTU, distilled water and a trace of HCl must be added to dissolve the substance. PhTU is dissolved in a mixture of benzene and methanol (8:92, v/v). DMTU is used as an internal standard : immediately before use dilute 0.5 ml of the stock solution to 100 ml with methanol.

Reference substances used :

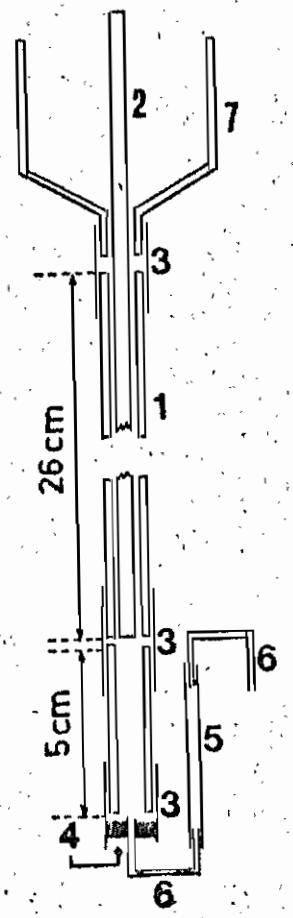
Symbol	Product
TAP	tapazole (2-mercapto-1-methylimidazole)
PTU	4(6)-propyl-2-thiouracil (4-hydroxy-2-mercapto-6-propylpyrimidine)
PhTU	4(6)-phenyl-thiouracil (4-hydroxy-2-mercapto-6-phenylpyrimidine)
TU	2-thiouracil (4-hydroxy-2-mercaptopyrimidine)
MTU	4(6)-methyl-2-thiouracil (4-hydroxy-2-mercapto-6-methylpyrimidine)
DMTU	4(5,6)-dimethyl-2-thiouracil (4-hydroxy-2-mercapto-5,6-dimethylpyrimidine)

3.15. Spiked sample containing 25 µg/kg anti-thyroid substances.
2.0 g of minced meat should be spiked by adding a methanolic solution containing 0.05 µg thiouracil, 0.05 µg methylthiouracil, 0.05 µg propylthiouracil and 0.20 µg tapazole. 0.05 µg dimethylthiouracil is added as an internal standard.

4. Equipment and accessories

Normal laboratory equipment and the following accessories.

4.1. Air- and water-tight reaction tubes, about 10 ml (e.g. sovirel tubes)
4.2. Micro-column



1. glass column (4 mm I.D., 6 mm O.D.)
2. glass rod (3 mm O.D.)
3. silicon tubing (4 mm I.D., 6 mm O.D.)
4. silicon tubing (0.5 mm I.D., 4 mm O.D.)
5. silicon tubing (0.5 mm I.D., 1 mm O.D.)
6. teflon tubing (0.3 mm I.D., 0.7 mm O.D.)
7. glass funnel

4.3. Homogenizer (e.g. Ultra-Turrax)
4.4. Water bath (40°C)
4.5. Equipment for thin layer chromatography
4.6. UV lamp 366 nm, with contrast filter in form of protection spectacles respectively
4.6. centrifuge
4.7. micro liter syringes with end of the needle cut at 90°, capacity 1 µl, 10 µl and eventually 100 µl

5. Procedure :

Extraction and clean-up

5.1. The meat must be in the fresh state (frozen or chilled) and at a temperature not exceeding + 7°C. 200 g of the sample are passed three times through a mincer (diameter of perforated plate : 2 mm). Storage for the first 24 h may be at 4°C ; for longer storage, the sample must be frozen at -20°C.
5.2. Take 2 g of minced tissue, transfer it to an extraction tube, add 10 ml methanol and homogenize using an Ultra-Turrax. Add 100 µl internal standard solution (3.14.) and centrifuge at 12 000 g during 10 minutes. Decant the supernatant layer.

5.3. Preparation of the micro-column for the clean-up of thyreostatic drugs : Place a plug of glasswool in the column, fill the column with water and remove the glass rod. Approximately 0.6 ml mercurated resin (3.11) is suspended in water and added to the glass funnel. After sedimentation of the resin in the column to a height of 5 cm, the excess of resin is removed. After depositing the glass rod on the resin bed, the column is ready for use.

NOTE : After use, the resin is discarded in order to avoid eventual cross-contamination.

5.4. Pour the methanol-water phase (5.2.) in the glass funnel of the micro-column (5.3.) and remove eventual air in the glass funnel by moving the glass rod in the column up and down. When the liquid has almost completely penetrated the resin, wash the column with 10 ml of water. Discard the effluents.

5.5. Place an extraction tube under the column exit. Then elute the thyreostatic drugs from the column with 5 ml of an acid-salt solution (3.5.).

5.6. Neutralize the eluate by adding 100 µl of 4 N NaOH (3.3.) and add 1 ml pH 8 buffer (3.9.). Eventually check the pH by means of a pH meter and adjust if required, to pH 8.0.

Reaction :

5.7. Add to the buffered eluate (5.6.) 0.1 ml NBD-Cl solution (3.10.). Mix (e.g. Vortex) and place the reaction tube in a closed water bath for one hour at 40°C (The reaction takes place in the dark). The optimum reaction time may vary from 60 to 120 min according to the quality of the NBD-Cl. This should be checked in advance, according to the NBD-Cl used.

NOTE : Reference solutions should be derivatized simultaneously with the samples (see 5.11.).

5.8. After the solution has cooled down, adjust the pH to between 3 and 4 by addition of 100 µl 7.5 N HCl (3.7.). Check the pH by means of a pH-meter and adjust eventually. Add 3 ml diethylether (3.8.) and shake the tube during a few seconds. After decantation, transfer the upper phase (ether), which contains the AH derivative, into a second extraction tube. Then extract the aqueous phase twice more with 2 ml of diethylether.

5.9. Add anhydrous Na_2SO_4 (0.5 to max. 1 g) to the combined diethylether phases. Shake, mix and stand 10 min. Decant the ether phase into a graduated centrifuge tube. Wash the sodiumsulphate with 2 ml diethylether and add it to the decanted ether phase.

NOTE : The presence of any water in the extract will invalidate the TLC as it will affect the development of the plates.

5.10. Evaporate down the ether phase (5.9.) under a current of N_2 to 0.1 ml. If two phases are produced, this indicates the presence of water and the extract should be diluted with diethylether and redried with anhydrous sodiumsulphate.

Derivatised reference solutions :

5.11. Prepare the reference solutions of the AH derivative by mixing 0,1 ml stock solution (20 µg) and 5 ml pH 8 buffer. Alternatively combined reference solutions of the AH derivations may be obtained by mixing 0.1 ml of each solution (20 µg) and 5.0 ml pH 8 buffer. Carry out the reaction and the extraction according to sections 5.7. - 5.9. Reduce the volume of ether to 2 ml.

6. Thin-layer chromatography on 10 x 10 cm plates (6.1. - 6.16.)

6.1. HPTLC-plates 10 x 10 cm (e.g. Merck n° 5547 or n° 5631) are used without preactivation. The plates used should show up at least 5 ng of a MTU reference solution in the form of well marked fluorescent spots after spraying.

6.2. Divide up the silica gel plate (6.1.) as indicated in Figure 1.

6.3. On to this plate place at the starting point S (Fig. 1) 10 µl of the concentrated extract (5.10). In channel A (see Fig. 1) overspot 5 µl of the sample extract with 1 µl of the derivatised reference solution of the AHs presumed to be present and check that the Rf values for the AHs are the same as when spotted alone. If not, an allowance must be made for the discrepancy when identifying the spots from the sample.

6.4. The chromatogram is developed following the first direction of migration using chloroform-ethanol (95:5, v/v) in a non-saturated tank.

6.5. After development to the required height, remove the plate and dry it carefully in a current of air.

6.6. If necessary, spot at the starting point (S) of the sample 0.5 µl of the AH or AHs presumed to be present. This method of procedure may be useful for additional Rf comparison between an unknown spot and the reference after the second development.

6.7. Then develop the plate in a direction perpendicular to the first direction of migration, using chloroform/propionic acid (95:5, v/v). After it has been developed, dry the plate carefully (remove the propionic acid before spraying the alkaline reagents !) in a current of air.

6.8. Examine the chromatogram at 366 nm and mark the fluorescence spots with a pencil circle. Absorbent black spots show the presence of substantial free or combined quantities of NBD-Cl (e.g. AH-NBD) on the plate.

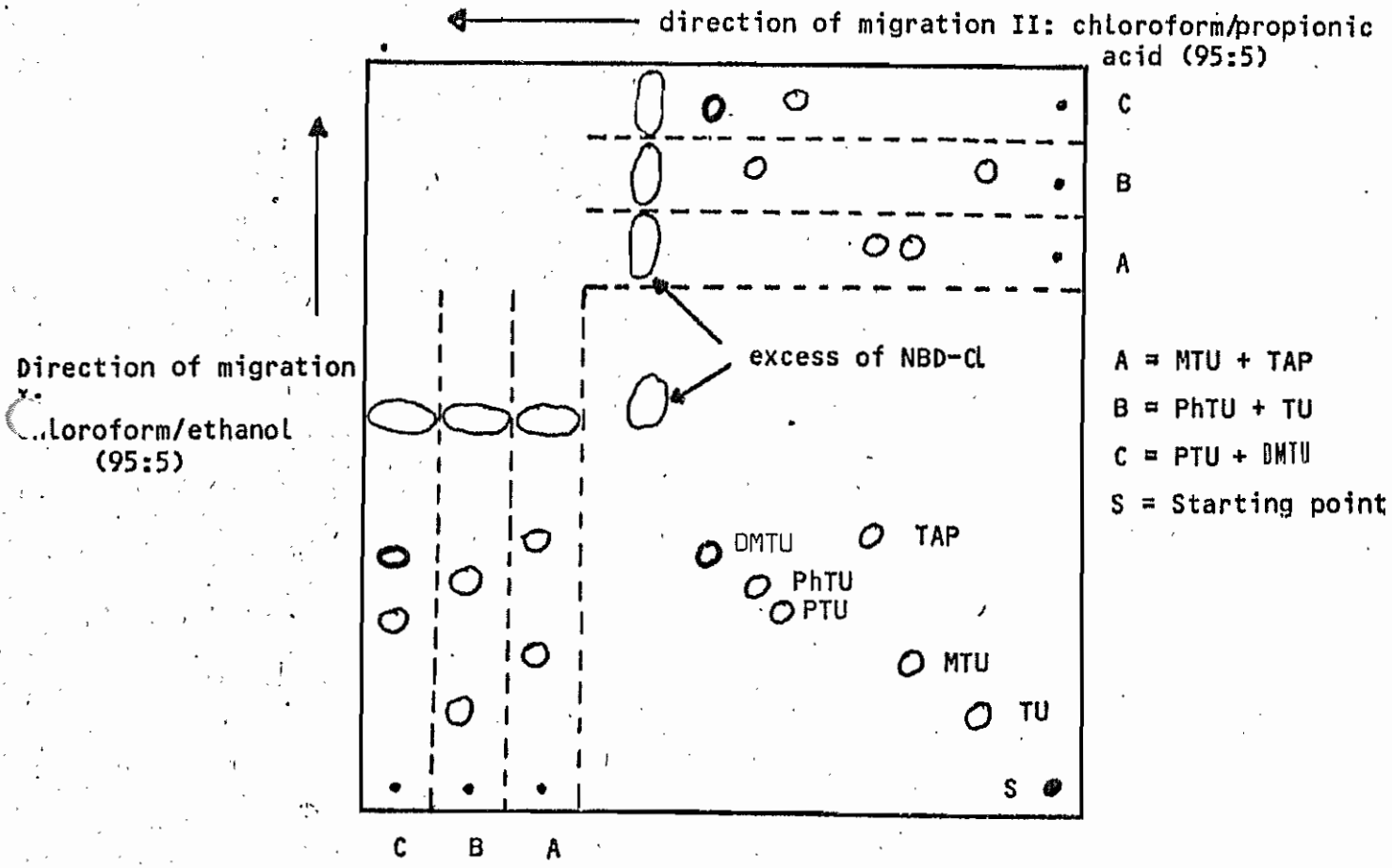
6.9. Spray the plate in a uniform manner using an excess of the spray reagent (3.13.) until it just appears wet. After drying re-examine the plate at 366 nm. Yellow fluorescent spots on a blue background, which were not visible before spraying, show the presence of AH.

6.10. Establish the identity of the anti-hormones by comparing the Rf values and the reference substances in the first and second directions of migration (see also 6.3.). The internal standard (DMTU) should be visible as a bright spot.

6.11. Confirm the identity of the AHs present in the sample by co-chromatography on a second plate. Place at the starting point S (Fig. 1) 10 µl of the concentrated extract (5.10.). Overspot the starting point S with 0.5 µl of the appropriate derivatised reference solution (5.11.). (Eventually, 0.5 µl of the derivatised reference solutions (5.11.) may be spotted in the lateral channels as indicated in Fig. 1). Proceed according to points 6.4-6.8. Observe that the AH(s) present in the sample coincide exactly with the reference derivative(s) added to the sample.

6.12. Interferences are reduced by leaving plates overnight.

Fig. 1 : Two-dimensional development



6.13. Low recoveries of DMTU in the sample as compared to DMTU in the reference solution (<70%) may be due to

i) incomplete elution of the AH from the micro-column (5.3.): reduce resin height

ii) inaccurate pH adjustment during NBD-reaction (5.7.) or extraction of NBD-derivatives (5.8.).

Table 1. :

Rf-values of thyreostatics on HPTLC-silicagel plates (10 x 10 cm) with some solvent system. (migration distance of front : 5 cm).

Solvent mixture (v/v)	Rf - value x 100					
	MTU	TAP	TU	PhTU	PTU	DMTU
1. chloroform-ethanol (95:5)	32	67	21	45	42	53
2. benzene-dioxane (65:35)	35	36	26	-	48	55
3. light petroleum-tetrahydro-furan-water (60:40:0.1)	28	19	16	-	52	76
4. dichloromethane-methanol (97:3)	32	87	20	-	42	60
5. chloroform-propionic acid (95:5)	48	58	34	76	68	73
6. dichloromethane-propionic acid (98:2)	16	18	11	-	29	31
7. dichloromethane-formic acid (98:2)	37	2	33	-	47	48
8. light petroleum-chloroform-diethylether-propionic acid (30:20:5:2)	23	8	16	38	47	-

- : Rf-value not determined

Note : for bidimensional development use : in first direction one of solvents 1 - 4
in second direction one of solvents 5 - 8.

7. Thin-layer chromatography on 20 x 20 cm plates -

7.1 Activation of the thin-layer plates. The best results were obtained with silica gel 60 plates (e.g. Merck n° 5745). The plates are previously activated by being heated for an hour at 110°C. The plates used should show up at least 20 ng of an MTU reference solution in the form of well marked fluorescent spot.

7.2. Divide up an activated silica gel plate (6.1) with a soft pencil as indicated in Figure 1.

7.3. On to this plate place at the starting point S (Fig. 1) 25-50 µl of the concentrated extract (5.14). In channel A (see Fig. 1) overspot 10 µl of the sample extract with 5 µl derivatised reference solutions of the AHs presumed to be present and check that the Rf values for the AHs are the same as when spotted alone. If not, an allowance must be made for the discrepancy when identifying the spots from the sample.

7.4. The chromatogram is developed following the first direction of migration using chloroform-ethanol (95:5, v/v) in a non-saturated tank.

7.5. After development to the required height, remove the plate and dry it carefully in a current of air.

7.6. If necessary, spot at the starting point (S) of the sample 2.5 - 5 µl of the AH derivatives presumed to be present. This method of procedure may be useful for additional Rf comparison between an unknown spot and the reference after the second development.

7.7. Then develop the plate in a direction perpendicular to the first direction of migration, using chloroform/propionic acid (95:5, v/v) in a non-saturated tank. After it has been developed, dry the plate carefully (remove the propionic acid before spraying the alkaline reagents !) in a current of air.

7.8. Examine the chromatogram at 366 nm and mark the fluorescent spots with a pencil circle. Absorbent black spots show the presence of substantial free or combined quantities of NBD-Cl (e.g. AH-NBD) on the plate.

7.9. Spray the plate in a uniform manner using an excess of the spray reagent (3.13) until it just appears wet. After drying ... re-examine the plate at 366 nm. Yellow fluorescent spots on a blue background which were not visible before spraying, show the presence of AH.

7.10. Establish the identity of the anti-hormones by comparing the Rf values with the reference substances in the first and second directions of migration (see also 6.3.). The internal standard (DMTU) should be visible as a bright spot.

7.11. Confirm the identity of the AHs present in the sample by co-chromatography on a second plate. Place at the starting point S (Fig. 1) 50 µl of the concentrated extract (5.14). Overspot the starting point S with 2.5 µl of the appropriate derivatised reference solution (5.15). (Eventually, 5 µl of the derivatised reference solutions (5.15) may be spotted in the lateral channels as indicated in Fig. 1). Proceed according to points 6.4-6.8. Observe that the AH(s) present in the sample coincide exactly with the reference derivative(s) added to the sample.

7.12. Interferences are reduced by leaving plates overnight.