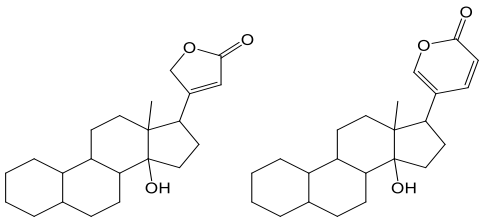
Practice No. 4

ANALYSIS OF DRUGS CONTAINING CARDIOACTIVE GLYCOSIDES

Cardioactive glycosides are a family of natural glycosides that have a specific effect on the activity of heart muscle; in lower doses, they decrease the frequency and increase the intensity of the heart contractions, in higher doses, they stop the heart.

The chemical structure of cardioactive glycosides consists of three parts – the steroid skeleton, the lactone ring and the sugar. The presence of the unsaturated lactone ring is essential for the activity. Cardioactive glycosides are divided into two basic groups according to the aglycone structure - c*ardenolides* and *bufadienolides*.



Cardenolides Bufadienolides

The sugar part is usually glucose, rhamnose and an array of deoxy sugars e.g. digotalose, digitoxose etc.

Identification

Color reactions are used. The most common reaction with low specifity is dissolving cardioactive glycosides in slightly diluted sulfuric acid (84%). This reaction is characteristic for many glycosides and aglycones e.g. it is used for sorting different species of the genus *Strophantus*. Other reactions used for the identification of cardioactive glycosides can be divided into 3 groups.

1- Reactions of cardenolides

Requirement is presence of double bond in lactone cycle of aglycone part of molecule. Reactions are provided in basic terms. There are following reactions:

Baljet reaction

Basic solution of picric acid forms light orange to dark red color. The presence of acetone interferes in the reaction and gives a false positive.

Raymond reaction

Using *m*-dinitrobenzene. It forms a purple-red color.

Kedde reaction

Using 3,5-dinitrobenzoic acid, it forms a purple-red complex.

Legal reaction

Using a diluted pyridine solution, it forms a red color with sodium nitroprusside.

2- Reactions of the sugar part

The Sugar component is separated by hydrolysis, and then it can react with several reagents (xanthydrol, vanillin, anthrone, o-dialdehyde of phthalic acid, *p*-dimethylaminobenzaldehyde) to form color products. The most known reaction, Keller-Kiliani, is used to prove the presence of 2-deoxysugars (D-digitoxose, D-cymarose). The sugars react with xanthydrol in the presence of HCl to form a red color.

3- Reactions of steroids

These reactions are based on the dehydration of the aglycone and its isomerization to form a colored complex.

Liebermann reaction

With acetic anhydride and concentrated sulphuric acid.

Rosenheim reaction

Using 90 - 98 % trichloroacetic acid. This reaction is characteristic for steroids with conjugated system of double bonds, or for aglycones that can create this system (bufadienolides).

Drugs that contain cardioactive glycosides

Digitalis purpureae Folium

Digitalis purpurea, Scrophulariaceae

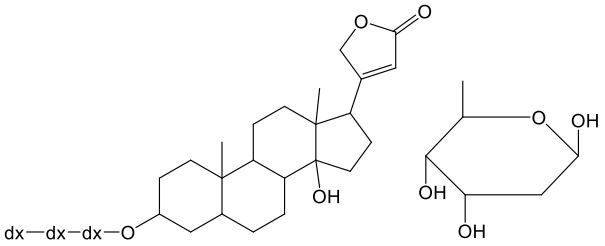
Dried leaf of *Digitalis purpurea* L.

Content compounds: min. 0,3 % of cardenolides, mainly purpureaglycoside A and B, which are decomposed upon hydrolysis into sugars and digitoxin ang gitoxin, saponins and tannins

Digitalis lanatae folium

Digitalis lanata, Scrophulariaceae

Content compounds: the same as Folium digitalis purpurea and lanatosides, mainly A and C.



Digitoxin dx=β-D-digitoxose

Thin layer chromatography

Test solution: 1.0 g of the powdered drug is boiled with 20 ml of 50% ethanol and 10 ml of lead acetate solution for 2 min. Exctract is filtered after cooling down and extracted two-times by 15 ml of chloroform in a separatory funnel. The united chloroform layers is dried by 1 g of anhydrous Na_2SO_4 and filtered again. 10 ml of the filtrate is vaporated until dryness in a water bath, the rest of the solution is left for the identification. The residue is dissolved in 1 ml of a mixture prepared from equal volumes of methanol and chloroform.

Lead acetate solution: solution of lead acetate (95 g/l) in water without CO_2

Reference solution: solution of digoxin, digitoxin and gitoxigenin

Elution mixture: water : methanol : ethylacetate (7.5 : 10 : 75)

Detection reagent: mixture of chloramine T solution (10 g/l) and trichloroacetic acid in 96% ethanol (250 g/l) in ratio 2 : 8

20 µl of both solutions are apllied on the TLC plate and eluted for a distance of 10 cm. The layer is dried and covered by the detection reagent. Then it is dried at 100 - 105 °C for 10 minutes, and then observed under UV light at 365 nm. On the chromatogram, there should be a light blue fluorescent spot of purpureaglycoside B on the lower part, and closely above that spot there should be a brown-yellow fluorescent spot of purpureaglycoside A. A light blue fluorescent spot of gitoxin should be found in the middle part and a brown-yellow fluorescent spot of digitoxin is above. There can be other visible fluorescent spots on the chromatogram.

Identification

1- 1 g of powdered drug is boiled with 15 ml of water and then is filtered using a cotton wool after cooling down. The filtrate is divided into two parts (solution A):

a) Four drops of lead acetate solution are added to the first part of a solution (solution A) until no further precipitate is formed and it is filtered. Sodium phosphate dibasic solution is added to the filtrate until no precipitation forms. The solution is filtered and 1 ml of freshly prepared Baljet reagent is added. The solution becomes orange-red in 20 minutes (cardioactive glycosides)

Baljet reagent: mix 95 ml of 1 % picric acid solution with 5 ml of 10% sodium hydroxide solution. It is diluted before use by methanol in ratio 1:1 immediately before use!!!

b) The 5 ml of chloroform, 1 ml of ether and 1 ml of ethanol are mixed with the second part of the solution (solution B) and it is shaken. The chloroform layer is evaporated on ceramic dish to dry. The residue is dissolved in 1.5 ml of conc. acetic acid, placed in a test tube and 1 drop of 0.1 % ferric chloride solution and 1.5 ml of sulfuric acid is added. Red-brown ring is formed at the interface (digitaligenines). The layer of acetic acid is changed into blue-green (digitoxose)

2- Proof of the presence of the sugar: 1 g of powdered drug is boiled with 15 ml of water and then is filtered using a cotton wool after cooling down. Four drops of lead acetate solution are added to the first part of a solution until no further precipitate is formed and it is filtered. Sodium phosphate dibasic solution is added to the filtrate until no precipitation forms. The solution is filtered, divided to two parts (solution C, D) and a few drops of conc. HCl and 1 ml of anthrone are added to first part (solution C). The color is changed to brown-red. Add 1 ml *p*-dimethylaminobenzaldehyde to another acidified filtrate. After heating, the orange-red color is formed.

3- Proof of the presence of the steroid part: A few drops of conc. HCl are added to the solution D and it is performed liquid-liquid extraction with 10 ml of chloroform.

Liebermann reaction: 3 ml of the chloroform extract is evaporated until dryness, 1 ml of concentrated sulfuric acid and 0.5 ml of acetic anhydride is added to the residue. Brown or green color is formed after 5 minutes.

Rosenheim reaction: 3 ml of the chloroform extract is evaporated until dryness and 1 ml of trichloroacetic acid is added to the residue. The solution becomes green.

4- 5 ml of the solution prepared for TLC is evaporated until dryness in a water bath. 2 ml of dinitrobenzoic acid and 1 ml of NaOH (1 mol/l) are added to the residue. In 5 minutes, the red-purple color is formed (Kedde reaction)

Dinitrobenzoic acid: solution (20 g/l) in 96% ethanol

5- 5 ml of solution prepared for TLC is evaporated until dryness in a water bath, 3 ml xanthydrol is added to the residue and heated in a water bath until a red color is formed

Xanthydrol solution: 100 ml of anhydrous acetic acid and 1 ml of hydrochloric acid are added to 0.1 ml xanthydrol (100 g/l) in methanol, add (the solution is prepared 24 hours before use).

Determination of content

A colorimetric method is used for quantitative determination of the cardioactive glycosides content in the drugs.

4.000 g of the finely powdered drug are place in a ground-glass flask, 50 ml of 70% warm ethanol is added and boiled under reflux for 15 minutes in a water bath, then it is left for 15 minutes to stand and cooled down to 20°C. The flask again is refilled using 70 % ethanol to the previous volume. 110 ml of water is added and mixed. 50 ml of 15% lead acetate solution are added and mixed it again. It forms a precipitate which settles down. Solution is filtered and 48 ml of 10% sodium phosphate dibasic solution are added to the transparent filtrate (160 ml of filtrate correspond to 3.2 g of drug). It is shaken and filtered again (162 ml of filtrate correspond to 2.5 g of drug). The filtrate is placed in a 300 ml separatory funnel and performed to liquid-liquid extraction successively with 50, 40, 40 and 40 ml of chloroform. The united chloroform extracts are evaporated using the vacuum evaporator until dryness.

The dry residue is dissolved in a mixture of chloroform - methanol (1 : 1) gradually to reach 25 ml. 2 ml of this extract is evaporated (200 mg of drug) until dryness and then the residue is dissolved in 35 ml of methanol. 5 ml of the methanolic solution is mixed with 5 ml of Baljet reagent. Absorbance is measured after 30 minutes at 494 nm. As a blank, a reference solution is used (5 ml of methanol + 5 ml of Baljet reagent).

The percentage of the amount of cardioglycosides calculated as lanathoside C can be calculated using this equation:

$\% = A_{1\,cm} * 3.41$ $A_{1\,cm}$.... measured absorbance

Herba convallariae

Convallaria majalis, Liliaceae

Content compounds: more than 20 glycosides, cardenolide type (mainly convalatoxin, convalatoxol etc.), saponins, derivates of flavones, small amounts of essential oils

Identification

Boil 1.1 g of the drug with 15 ml of water and filter the decoction using a piece cotton wool. Divide the filtrate into two parts.

a) Add 5ml of chloroform, 1 ml of ether and 1 ml of 95% ethanol to the first part of decoction and shake thoroughly. Evaporate the chloroform layer on ceramic dish until dryness and dissolve the residue in 1.5 ml of concentrated acetic acid. Then add 1 drop of 0.1% ferric chloride solution, cover 1.5 ml of sulfuric acid in a tube by that solution. A Red-brown ring is formed at interface of the two layers (cardioactive glycosides).

b) Add four drops of lead acetate solution to the second part of the filtrate until no further precipitation forms and filter. Add sodium phosphate dibasic solution to the filtrate until no precipitation forms. Filter the solution again, and add an equal volume of freshly prepared Baljet reagent. The solution becomes red within 30 minutes (cardioactive glycosides)

Determination of content

3.000 g of the finely powdered drug is placed in a ground-glass flask and 50 ml of 70% warm ethanol is added. The mixture is boiled under reflux condenser for 15 minutes in a water bath, then it is left for 15 minutes to stand and cooling down to 20°C. 70 ml of water and 20 ml 20% lead acetate solution are added and they are mixed. It forms precipitations which settle down. Solution is filtered and 25 ml of 10% sodium phosphate dibasic solution is added to 100 ml of the transparent filtrate. It is shaken and filtered again. 100 ml of the filtrate (correspond to 2.0 g of drug) is placed in a 200 ml separatory funnel and performed liquid-liquid extraction successively with 60, 35, 35 and 35 ml of chloroform. The united chloroform extracts are evaporated using vacuum evaporator until dryness. The residue is dissolved in 10 ml of methanol and the solution is filtered. 1 ml of the methanolic solution is used and completed the volume to 10 ml by methanol. 5 ml of this solution is mixed with 5 ml of Baljet reagent. Absorbance is measured after 15 minutes at 494 nm, as a blank, use a reference solution (5 ml of methanol + 5 ml of Baljet reagent).

The percentage of the amount of cardioglycosides calculated as convalatoxin can be calculated using this equation

$\% = A_{1\,cm} * 0.625$ $A_{1\,cm}$ .... measured absorbance

Semen strophanti

Strophanthus gratus, Apocynaceae

Content compounds: cardenolides (mainly strophanthin), saponins, choline, proteases and oils

Identification

1- 5 ml of water is added to 0.5 g of the powdered drug, the mixture is boiled and filtered after cooling down. An equal volume of the freshly prepared Baljet solution is added to the filtrate. The color of the solution becomes orange-red in 30 minutes (cardioactive glycosides)

2- A drop of 80% sulfuric acid is added to a thick slice of the seed in a watch glass, the color change according to the species of *Strophanthus* is observed:

Strophanthus courmontii	- brown to green color after 10 minutes.
Strophanthus eminii	- brown to purple color after 5 minutes
Strophanthus gratus	- pale red, later red to red-purple color
Strophanthus hispidus	- brown-red color
Strophanthus kombé	- intense dark green color
Strophanthus nicholsonii	- brown color that changes in 10 minutes into purple
Strophanthus sarmentosus	- pale red