ÚSTAV LÉKAŘSKÉ BIOCHEMIE A LABORATORNÍ DIAGNOSTIKY 1. LF UK A VFN

Toxicology

Practical lesson in medical biochemistry

General Medicine

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2024/2025

Task 1: Thin-layer chromatography of selected drugs

Reagents:

1. **Ethyl acetate** 
2. **Methanol** 
3. **Marquis Reagent** 
   4 ml 40% formaldehyde added to 100 ml 96% sulfuric acid.
4. **Mandelin Reagent** 
   1.0 g of ammonium metavanadate dissolved in 100 ml of 96% sulfuric acid
5. **Dragendorff Reagent** 

   Solution A: 6.0 g of potassium iodide dissolved in 10.0 ml of deionized water.

   Solution B: 0.6 g of alkaline bismuth(III) nitrate dissolved in 31% hydrochloric acid; then 10.0 ml of deionized water is added.

   Solution C: 8.0 ml of 31% hydrochloric acid combined with 475 ml of deionized water.

   Full volumes of solutions A, B, C are gradually mixed to obtain the final reagent.
6. **Iodine** 
7. **Samples of drugs**:

   Furosemide 
   Solution for inj. 10 mg/ml

   Diclofenac 
   Solution for inj. 25 mg/ml diluted with 96% ethanol 1:2

   Tramadol 
   Solution for inj. 50 mg/ml diluted with 96% ethanol 1:4

   Mirtazapine 
   Oral pill with 30 mg of mirtazapine allowed to disintegrate in 3 ml of mixture ethyl acetate : methanol : deionized water 9:6:1, undissolved content is removed by centrifugation.

   Metoprolol: 
   Oral pill with 100 mg of metoprolol allowed to disintegrate in 5 ml of mixture ethyl acetate : methanol : deionized water 9:6:1, undissolved content is removed by centrifugation.

Tools and equipment:

1. Sheets for thin layer chromatography Alugram® SIL G UV_{254}, 10 × 15 cm
2. Chromatographic chambers
3. UV lamp or transilluminator
4. Sprayers for detection reagents
5. Fume chamber
6. Incubator 95 °C

Procedure:

Each pair of students will run one chromatographic sheet and perform a sequential detection with Marquis, Mandelin and Dragendorff reagent.

a) Prepare a mobile phase: to chromatographic chamber measure 30 ml ethyl acetate and 20 ml methanol. Cover the chamber immediately with glass lid, mix well by shaking and left it closed for at least 15 minutes to saturate the inner space with solvent vapors.

b) Prepare the sheet for thin-layer chromatography: with a soft pencil (not pen!) mark the start as a line along a shorter side of the sheet, about 1 cm from the edge. Make 5 points on the line in roughly equal distances about 1.5 cm from each other and about 2 cm from the margins. At the opposite edge mark which sample will be applied to each position.

c) Apply the samples of drugs (5 µl each) in the following order:

1: furosemide
2: diclofenac
3: tramadol
4: mirtazapine
5: metoprolol

Keep the sample vials closed and always take a new clean pipette tip for a new sample! Wait until the applied samples get completely dry.

d) Place the sheet into the chromatographic chamber with mobile phase. Allow the chromatograms to develop 10-20 minutes.

e) When the mobile phase has reached at least two thirds of the sheet, remove the chromatogram and immediately mark the position of the solvent front with pencil.

f) First step in detection consists of placing the chromatogram (without drying) under the UV lamp. Inspect the sheet both under UVA and UVC and mark any fluorescent spots with pencil, or take a picture of the sheet.

g) Next, in the fume hood spray the (still wet) chromatogram with the Marquis reagent (Caustic!). Spray only lightly and gently from a distance, avoid washing out the chromatogram with the reagent! Bluish spot of tramadol will appear. Place the sheet to 95 °C for 3 minutes. Record the presence and appearance of all spots in the visible light, UVA and UVC.

h) Spray the sheet with Mandelin reagent and heat in 95 °C until violet spot of metoprolol becomes visible (about 3 minutes). Review the sheet and record the presence and appearance of all spots in the visible light, UVA and UVC.

i) Finally, spray the sheet with Dragendorff reagent. Mirtazapine spot turns orange. Heat the sheet in 95 °C for about 1 minute – the background fades, but the mirtazapine spot should still be evident. Evaluate the sheet again and record position and appearance of all spots.

Evaluation and conclusion:

Draw a scheme of the chromatogram and calculate R_f for all the detected spots.

Note: You can keep the chromatogram, however, aluminium from the Silufol sheet replaces hydrogen of the detection reagent, Al_2O_3 and H_2 are formed and chromatogram quickly disintegrates.

Task 2: Proof of ethanol by reaction with potassium dichromate

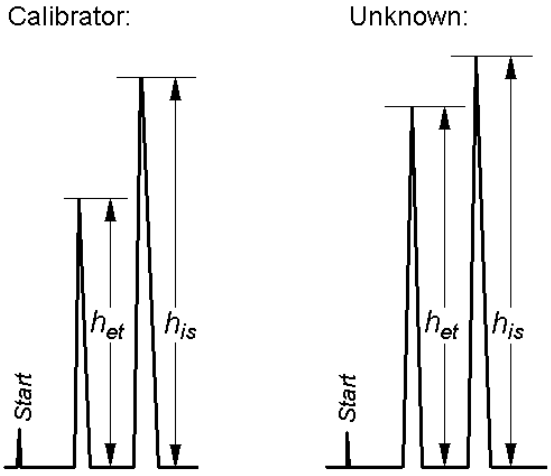
Reagents:

1. Potassium dichromate, $K_2Cr_2O_7$, solution, 3 g/l 
2. Ethanol 
3. Sulfuric acid, concentrated 

Procedure:
Combine about 0.5 ml of $K_2Cr_2O_7$ solution with equal volume of ethanol and add carefully (plastic shield is recommended) 5-6 drops of concentrated sulfuric acid. Within 1-2 minutes the solution turns green.
The reaction products should be collected to bottles for toxic waste.

Task 3: Estimation of ethanol in blood by means of gas chromatography – evaluation of chromatographic trace

Procedure:
You are given an authentic recording from gas chromatography on ethanol. First record represents analysis of the ethanol calibrator together with the internal standard (isopropanol); next comes analogous analysis of an unknown sample. Using a ruler, measure (in mm) peak height of ethanol and isopropanol both in the calibrator and the unknown sample, and calculate concentration of ethanol in the latter.



Calculation:

$$c_{et}\ \textit{in the sample} = \frac{\left(\frac{h_{et}}{h_{is}}\right)_{SAM} \times \left(\frac{h_{is}}{h_{et}}\right)_{CAL} \times c_{st}}{k}$$

c_{et}	concentration of ethanol in the unknown sample (in ‰)		
c_{st}	concentration of ethanol in the calibrator (in ‰ , written at the chromatogram)		
$(h_{et})_{SAM}$	peak height of ethanol in the unknown sample		
$(h_{et})_{CAL}$	peak height of ethanol in the calibrator		
$(h_{is})_{SAM}$	peak height of isopropanol in the unknown sample		
$(h_{is})_{CAL}$	peak height of isopropanol in the calibrator		
k	coefficient	whole blood, urine	$k = 1.0$
		hemolytic serum	$k = 1.1$
		serum	$k = 1.2$

(Ethanol distributes preferentially into the aqueous compartment of blood, and because the water content of serum (~ 98%) is greater than that of whole blood (~ 86%), coefficient k has to be used in calculation. For your work let's assume the sample was the whole blood).

Evaluation and conclusion:
Conclude what is concentration of ethanol in the evaluated sample according to its GC recording.

Task 4: Detection of ethanol with breathalyzer (Demonstration)

Tools:
1. Alcohol tester
2. Mouthpieces

Task 5: Demonstration of rapid immunochromatography test for detection of drugs in urine

Tools:
1. Rapid immunochromatography tests for detection of drugs in urine
2. Sample containing the detected substance
3. Control sample without the detected substance

Task 6: Spectrophotometric examination of hemoglobin derivatives

Reagents:

1. Potassium ferricyanide $K_3[Fe(CN)_6]$
2. Sodium dithionite $Na_2S_2O_4$ 
3. Undiluted heparinized blood (**Infectious material**)
4. Undiluted heparinized blood saturated with carbon monoxide (**Infectious material**)

Procedure:

Using the diode array spectrophotometer, record the absorption spectra of the following hemoglobin derivatives in the **wavelength range 500 – 600 nm**. Distilled water serves as a blank.

Oxyhemoglobin (O_2-Hb)
It prevails in the blood samples standing in the air. Add 0.02 ml of the blood to 4.0 ml of distilled water, mix, and record the spectrum. If the absorbances are still too high, the sample can be diluted further.

Deoxyhemoglobin (Hb)
Prepare by reducing the oxyhemoglobin solution (diluted as above) with a small amount (tip of spoon) of sodium dithionite; after 2-5 minutes record the spectrum. Compare with the values for O_2-Hb. Notice also any difference in color of the solution.

N.B.: *Only a really small amount of dithionite must be added, otherwise the protein precipitates – in that case prepare a new mixture for the measurement. On the other hand, if you do not see the expected change, i.e. the two peaks of oxyhemoglobin replaced with a single peak of deoxyhemoglobin, record the spectrum again later and/or try to add a bit more dithionite.*

Methemoglobin (hemiglobin)
To 4.0 ml of distilled water add 0.02 ml of blood and also a small amount (3-4 grains) of potassium ferricyanide as the oxidation reagent. After 5-10 minutes record the absorption spectrum and compare with the values for hemoglobin. Notice also change in color.

N.B.: *Again, add only a small amount of ferricyanide. If the solution has become yellow and/or precipitated, too much was added and it must be repeated. But if you do not see the expected change in spectrum, it means a longer incubation and/or more ferricyanide is needed.*

Carbonyl hemoglobin
To 4.0 ml of distilled water add 0.02 ml of blood saturated with CO. Evaluate the color and measure the spectrum. Check whether there is indeed the expected shift of the absorption maximum to 570 nm compared to oxyhemoglobin that has it at 578 nm. This shift is used for estimation of carbonyl hemoglobin in CO poisoning.

Evaluation:

In each recorded spectrum, find the absorbances for selected wavelengths as directed by the table in your lab report form. Fill these values to the table. Find also the absorption maxima and assess whether they match the theoretical expectations.

Use a piece of graph paper or Excel to draw the absorption spectra of the hemoglobin derivatives.