Date ..... Name ..... Group .....

Instructions for the practical lesson on biochemistry

Topic: Selected examinations in toxicology

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:

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 from each other and 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! Dry all the starts with a hair dryer.

d) Place the sheet into the chromatographic chamber with mobile phase. Leave 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 solvent front with pencil.

f) First step in detection consists of placing the chromatogram (without drying) under the UV light (366 nm). Mark any fluorescent spots with pencil.

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.

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.

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.

Alternatively, it is possible to place the sheet right after chromatography to a closed chamber containing flakes of elemental iodine. Leave the chromatogram inside until the iodine vapors turn its surface to yellowish brown. Then remove the sheet and immediately close the chamber again. Record the 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:

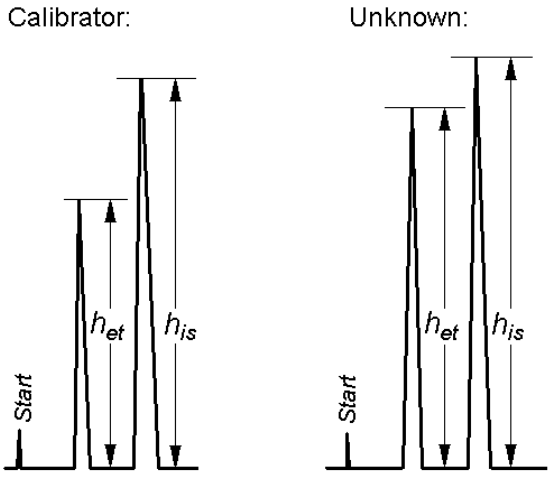
Combine about 0.5 ml of $K_2Cr_2O_7$ solution with equal volume of ethanol and add carefully (plastic shield is recommended) 5-6 drops of concentrated sulfuric acid. Within 1-2 minutes the solution turns green.

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.