PHG 454

lab #

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Detection of Abused Drugs

(using T.L.C. in urine sample)

PHG 454 Practical course

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Background:

Thin Layer Chromatography (TLC) , and what is its principle?.

Objectives :

- To understand the methods of abused Drugs analysis or test.
- To understand the TLC Techniques and its principle.
- To know some physical and chemical properties of Abused Drugs
- To learn some analytic laboratory skills.

1.2 Introduction:

Urine drug testing is the most common way to test for drugs. However there are a number of different drug testing methods available and sometimes the hair, saliva or perspiration may be drug tested.

To understand the principles behind drug testing technology, some knowledge is required about the way in which the body deals with chemical compounds such as drugs (PHARMACOKINATIC of DRUGS). Irrespective of the method of intake, all drugs, pharmaceutical and otherwise, undergo a series of bio-chemical reactions in the body. These reactions release the active compound and then gradually degrade the drug into slightly different structures. These structures, (metabolites), are then excreted from the body in a variety of ways. Urine is the main excretory route for drugs and their metabolites.

❖ URINE DRUG TESTING

The following is a summary of the analytical methods used by laboratories to detect the presence of drugs or their metabolites in urine.

Immunoassays

These tests are most commonly used to screen samples. In the event that drugs or their metabolites are detected, then the sample is normally tested again using an even more sensitive test such as Gas Chromatography and Mass Spectrometry. Immunoassays work on the principle of antigen-antibody interaction. Antibodies are chosen which will bind selectively to drugs or their metabolites. The binding is then detected using either enzymes, radioisotopes or fluorescent compounds.

Thin Layer Chromatography

This procedure involves the addition of a solvent to the sample causing the drugs and their metabolites to travel up a porous strip leaving color spots behind. As each different substance travels a specific distance, the strip can then be compared with known standards. This test gives no quantities information, it merely indicates the presence of drugs or their metabolites. Furthermore, it relies on the subjective judgment of a technician and requires considerable skill and training. It is not widely used.

Gas Chromatography and Mass Spectrometry

These are the most precise tests for identifying and quantifying drugs or their metabolites in the urine. They are usually used as a confirmation test following a positive result on an Immunoassay. It involves a two step process, whereby Gas Chromatography separates the sample into its constituent parts and Mass Spectrometry identifies the exact molecular structure of the compounds. The combination of Gas Chromatography and Mass Spectrometry is considered to be the definitive method of establishing the presence of drugs or their metabolites in the urine. However, the equipment necessary to perform it is extremely expensive and this is reflected in the price for testing each sample. Occasionally problems do arise with poor calibration of the equipment.

SPECIMENS.

The preferred specimen for drug testing now is urine, although alternative specimens such as hair, oral fluids (saliva), and sweat have potential (see Table I). The half-life of drugs in blood is short, making blood specimens less useful for routine drug screening (though a high concentration of active drug will suggest recent usage and, sometimes, likelihood of impairment).

Sample	Advantages	Disadvantages
Blood	◆Can be used to infer impairment ◆Difficult to adulterate	◆Short half-life of drugs ◆Requires phlebotomy ◆Low drug concentrations
Hair	◆Potential for long-term assessment of drug use	◆Requires difficult analytical procedures ◆Drug deposition not uniform among hair types ◆Testing is expensive
Saliva	◆Difficult to adulterate	◆Low drug concentrations ◆Difficult to get large volumes for confirmation

Urine	◆Noninvasive ◆Available in large volumes ◆Remains positive 2–3 days	◆High adulteration potential when collection not witnessed

Table I. A comparison of the types of samples that can be used in drugs-of-abuse testing.

HOW LONG AFTER USAGE CAN DRUGS BE DETECTED

There is no simple answer to this question, because it depends very much on the substance itself and on other factors such as dosage, frequency of abuse, and individual rates of drug metabolism and excretion; also on the sensitivity of the particular test carried out (Table 2). As an approximate guide, drugs are likely to be detectable in urine within the time scales shown in Table 3.

Table 2. SENSITIVITIES OF URINE SCREENING

AND CONFIRMATION TESTS FOR DRUGS OF ABUSE

At concentrations below the limit of detection, the drug (or metabolite) will not normally be detected by the usual screening procedure. A result reported as 'positive' in a screening test implies that the concentration of drug present is in excess of the limit given here. Note that the sensitivity of drug detection will be reduced in dilute urine specimens.

DRUG CLASS	LIMIT OF DETECTION OF SCREENING TEST	LIMIT OF DETECTION OF CONFIRMATION TEST
Alcohol	100 mg/L (10 mg/dL)	100 mg/L (10 mg/dL)
Barbiturates	200 µg/L	200 µg/L
Amphetamine	1000 µg/L	200 µg/L
Benzodiazepines	200 µg/L	200 µg/L
Cannabinoids	50 µg/L	5 µg/L
Cocaine (metabolite)	300 µg/L	50 µg/L
Codeine	300 µg/L	50 µg/L
Dihydrocodeine	300 µg/L	50 µg/L
6-Monoacetyl morphine		10 µg/L
Morphine	300 µg/L	50 µg/L

The action limits used by the laboratory for employment and pre-employment confirmatory tests cited above are the same as those used by most other UK laboratories performing drugs of abuse screening, and conform to the Substance Abuse and Mental Health Services Administration (SAMHSA) guidelines. In addition, the EU and / or UK guidelines can be applied to screening and confirmatory tests performed. Please contact the laboratory for further information.

Table 3. APPROXIMATE DETECTION TIMES OF SOME COMMON DRUGS OF ABUSE IN URINE*

DRUGS	DURATION OF DETECTION IN URINE:
Alcohol	up to 1 day
Amphetamines	1-3 days
Barbiturates	1-3 days
Benzodiazepines	1-3 days
Cannabis	up to 14 days
Cocaine	1-3 days
Codeine	1-2 days
Dihydrocodeine	1-2 days
Heroin (morphine)	up to 1 day
Methadone	1-3 days

◆ Note that detection times are only very approximate and are very dependent upon the dose, its frequency, route of administration and urine excretion/dilution.

2.2 Experement :

Sample used :

Different urine samples containing one or more of the following drugs:

- ☒ Morphine
- ☒ Cocaine
- ☒ Amphetamine
- ☒ Phenoparbitone

🗗🗗 These sample can be prepared as 0.1% in dilute acids for lab use .

Principle of assay :

Different drug in a urine sample can be extracted by organic solvent(s) at certain PH . The drug(s) in the concentrated solvent extract can be identified by thin layer chromatography (TLC) .

Procedure :

A. Preparation of Sample:

1) Transfer, about 10ml of the urine sample into a 100 ml separatory funnel.
2) Add few drops of bromocresol purple reagent , until the sample acquires the same reagent color (purple) .
3) Adjust the PH to about 8 , using few drops of ammonia . The sample turns violet.
4) Extract the drug(s), twice each 20 ml of chloroform/propan-2-ol (3:1), receive the combined extract in another separatory funnel. containing about 5 ml of ammonia chloride (PH 9.4) , mix well.
5) Receive the lower organic solvent in a 100ml conical flask , through anhydrous sodium sulphate (Dehydration) , distill of the solvent .
6) Dissolve the residue in 1-2 ml of $CHCl_3$/MeOH and apply for TLC .

B. TLC of the separate compounds :

◈ Sample Applicators

Fine capillaries for applying spots can be made from pasteur pipets. Your demonstrator will assist you in this.

Developing Chamber

Use the chromatography jar provided with its cover. The developing solvent system is a mixture of:

Ethylacetate:MeOH:Ammonia (17 : 2 : 1)

Place 10 mL of this solvent mixture (which is provided to you) in the chromatography jar, cover tightly and allow to stand 5 - 10 minutes before using.

◈ Application of the Samples

Use the commercially prepared fluorescent Silica Gel TLC sheet, supported on glass plate, 10 x 5 cm.

You will have five solutions (4 reference compounds and 1 sample) to examine. They should be spotted on the coated side in a line 1 cm from one end of the plate, equally spaced apart, with the outer two spots about 0.75 cm from the edge of the plate. The sample should be placed in the centre, with two reference compounds on each side.

To apply a sample, touch the rounded end of a melting point tube to the solution, and then gently touch the Silica Gel plate at the proper spot. Use a fresh melting point tube for each sample. The sample spots should not be larger than 2 - 3 mm diameter.

◈ Development of the Chromatogram

When each plate has been prepared, place the plate, spotted end down, in the developing jar. Make sure that the solvent pool begins below the spots. Cover tightly, do not disturb, and allow for the solvent to rise to within about 1 cm from the top of the plate - do not allow the solvent to run all the way to the top of the plate! Remove the plate and immediately mark the solvent front. Allow the sheet to dry.

◈ Visualization

The colourless compounds are visualized by illumination of the plate with an ultraviolet lamp. Many substances, particularly aromatic compounds, will show a bright fluorescence, which may have a characteristic colour. The thin layer plates used contain a trace of fluorescent dye. Compounds which are fluorescent show up as bright spots on a light background; any others appear as a dark spot since they quench the fluorescence of the background dye. Circle the spots lightly in pencil, and note any distinctive colours. In This experiment you have visualized by UV at 254 nm.

Then spray with Dragendorf reagent (which is a specific reagent for alkaloids)

◈ Results

(a) Calculate the Rf values of the reference compounds and the components of the sample.

(b) Draw the chromatogram to scale in your lab report; identify and label the spots in the chromatogram, including as many of the spots in the unknown as possible.

(c) From the number, position and appearance of the spots in the sample, and the composition of the possible unknowns, identify your unknown drugs.

CONDITIONS :

- Stationary phase : Silica gel 60 F_{254} (5X10)
- Mobile phase : Ethylacetate:MeOH:Ammonia (17:2:1)
- Detection : by UV at 254 nm then spray with Dragendorf reagent
- References : Cocaine , morphine ,Phenobarbital and Amphetamine dissolved in $CHCl_3$: MeOH .

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Notes on the Procedure :

In case that the ultraviolet lamp is not available , the following changes in the conditions can be done for TLC :

1. For morphine and cocaine solvent system will be :
   Ethyacetate : MeOH : NH_4OH (17:2:1) .
   And spray with iodoplatinate .

2. For amphetamine :

Using plate pre-treated with 0.5 N NaOH:MeOH
and the solvent system is MeOH:NH_4OH (100:1.5)
and spray with iodine/chloroform (1%) or using iodine chamber .

3. For Phenobarbitone :

Same with amphetamine but the detector is $HgSO_4$.

Reagent Used :

- Bromocresol/purple

  It gives yellow with weak acid solution and bluish-violet in alkaline and neutral media , and extremely weak acids (5.6 – 6.8 PH) .

  PREPARATION :

  Warm 0.1 g bromocresol purple with 5 ml 95%EtOH until dissolved , add 100 ml of 20 % EtOH ,3.7 ml of 0.05 N aqueous NaOH solution and complete to 250 ml with 20 % EtOH .

- Ammonium chloride (PH 9.4) :

  Prepare 10% aqueous solution then adjust the PH to 9.4 using the PH meter, using conc. ammonia .

- Mercuric sulphate solution ($HgSO_4$) :

  Weigh 5 gm yellow mercuric oxide (HgO) , add 40 ml H_2O then (slowly and cautiously) add 20 ml conc. H_2SO_4 , stir until dissolved , leave to cool , then complete to 100 ml with H_2O .

- Iodoplatinate reagent :

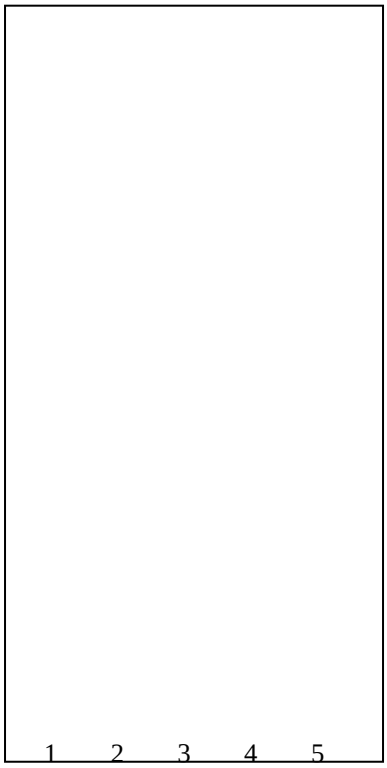
  Mix 3 ml of 10% hexachloroplatinic acid (IV) solution with 97 ml H_2O , add 100 ml 6% aq. Potassium iodate solution , keep in dark .

DRUG ABUSE DETECTION REPORT :

Examiner: **ID:**
Date:

- Sample Code: .....
- Sample type: .....
- Conditions:

1. .....
2. .....
3. .....
4. .....



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1.	4.
2.	5.
3.	6.

- Rf value (or other Note)

Spots Code	Rf value	Color	Notes

- Conclusion :

- Final Result(s):

Homework:

An unknown liquid sample is analyzed using paper chromatography using solvent X as the mobile phase. One spot is observed after the plate is developed and visualized. The same unknown substance is re-analyzed using solvent Y as the mobile phase. This time, three spots are observed after the plate is developed and visualized.

Is the unknown sample a pure substance or a mixture? Explain your answer, including a possible reason for the different observations in the two experiments