Urine analysis I: Chemical examination

General Medicine

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Urine is a biological fluid whose analysis provides valuable information about condition of the human body and its metabolic state. Examination of urine is one of the basic procedures in clinical chemistry that significantly contributes to diagnostic process, as well as monitoring of disease course and effects of therapy. Analysis of urine employs a wide array of techniques ranging from the simplest colored and precipitating test-tube reactions to very sophisticated and automated ones such as flow cytometry and computer analysis of urinary sediment. Test strips enable basic examination of urine not only in the laboratory, but also directly in the consulting rooms or at patient's beds in the hospitals.

This material describes the usual procedures of routine analysis of urine.

1. Collection of urine

In order to get valid results from urine analysis, an appropriate and correct technique of urine sample collection is essential.

1.1. Early morning urine

For majority of urine examinations the first early morning urine is the most suitable. It is recommended the urine collection is preceded by at least eight hours of lying position. The early morning urine is more concentrated and acidic compared to the later samples, and suitable especially for chemical examination. The urine taken later during the day is more affected by liquid intake, food and physical activity.

Urine is typically obtained by a ***spontaneous micturition***. The actual urine collection is to be performed after thorough washing and wiping of the external urethral orifice. A perfectly clean, dry, and capped vessel should be used; in particular, it must be free from detergents and disinfectants that distort chemical analyses. The middle flow of urine is best for analysis. The initial flow is always contaminated with cells and bacteria from around the external urethral orifice. Therefore, the patient should dispose the first flow or urine into closet, and pick up the following portion of urine into the collection vessel.

In certain situations it might be necessary to obtain the urine sample by ***percutaneous suprapubic puncture of urinary bladder***, or by ***urinary bladder catheterisation***.

In women the examination of urine is avoided shortly before, and shortly after the menstruation.

For majority of qualitative and semiquantitative chemical analyses with urine test strips, no stabilising additives into the urine sample are necessary. If, however, it is not possible to examine the urine sample within two hours since its collection, the urine should be kept refrigerated or chemically conserved.

During long standing of non-conserved urine the composition of urine changes:

Proliferation of bacteria	• *↓ glucose (degraded by bacteria)*
	• *↑ nitrites (reduction of urinary nitrates to nitrites)*
	• *Conversion of urea to ammonia → ↑ pH*
	• *Production of substance interfering with estimation of some analytes:*
	   ○ *false positivity of bilirubin*
	   ○ *false positivity of urobilinogen*
	   ○ *false positivity of ketone bodies*

Evaporation of volatile substances	• *↓ ketone bodies (especially acetone)*
Degradation of photosensitive substances	• *↓ bilirubin*
Oxidation with air oxygen	• *↓ urobilinogen*
Decomposition of particulate components (erythrocytes, leukocytes, casts)	

1.2. Second morning urine

It is a sample produced 2–4 hours after the first early morning urination. Its composition is affected by food intake, drinking and physical activity. The second morning urine is recommended chiefly for quantitative estimations related to urine creatinine.

1.3. Random urine sample

The random sample means a collection of fresh urine without knowledge of collection time, daily volume of urine, nor any details of previous patient's history. It is considered in emergency medicine. The urine analysis can produce false positive, as well as false negative results.

1.4. Time collection of urine

For quantitative analyses and estimation of clearance of various substances, the urine must be collected during a defined period of time. We can distinguish a ***short-term urine collection*** lasting 1–3 hours, and a ***long-term collection*** that takes 12–24 hours. Occasionally an overnight (8-hour) collection is performed, e.g. for estimation of microalbuminuria. The accuracy of urine collection is critical for results of the examination. It can be checked by measuring concentration of creatinine in urine.

The urine is collected into clean vessels placed in a cool and dark place; a preservative reagent may be added.

The collection can start any time during the day after emptying of the urinary bladder and recording its time. In case of 24-hour collection, however, a start in early morning between 6 to 7 o'clock is considered most suitable. Patient starts with urination; this portion of urine is not collected. Since that moment all urine is collected. Patient should remember to urinate to the collection vessel also before defecation. Exactly after 24 hours the collection ends with the last and complete urination into the sample vessel. The volume of all urine is measured (with precision to ml), the urine is mixed, and at least 5 ml sample is taken and transported to the laboratory, together with data about the time of start and end of the collection period (with precision to minutes), as well as about the precise volume of urine.

The basic examination of urine includes:
- **physical examination,**
- **chemical examination,**
- **morphological examination (urinary sediment)**

2. Chemical examination of urine

The routine chemical examination of urine involves qualitative tests for ***protein, glucose, hemoglobin, ketone bodies and bile pigments.*** These components are mostly present in urine from healthy individuals as well, but in tiny amounts undetectable by the routine tests. Various pathological conditions increase their concentration in urine.

2.1. Test tube reactions

In the past, colored/precipitating reactions performed as 'wet chemistry', i.e. in test-tubes, were used for detection of the pathologic components of urine. Some of these classical reactions have been already introduced in the previous practical lessons (which see), and their principles are summarized in table 1.

Table 1: Principles of detection of pathologic components in urine by means of the colored/precipitating test-tube reactions

Analyte	Principle of reaction	Particular tests
Protein	denaturation of proteins by acids or boiling	• test with sulfosalicylic acid • Heller's test (with concentrated HNO_3) • boiling test
Hemoglobin	iron in heme displays a pseudoperoxidase activity, which catalyses oxidation of chromogen with organic peroxide to a colored product	• Heitz-Boyer's test (oxidation of reduced phenolphthalein) • benzidine test (oxidation of o-tolidine or tetramethylbenzidine)
Glucose	non-specific tests based on reducing properties of glucose	• Fehling's test (reduction of Cu^{2+}) • Benedict's test (reduction of Cu^{2+}) • Nylander's test (reduction of Bi^{3+})
Ketone bodies	reaction with sodium nitroprusside in alkaline medium producing a violet complex	• Legal's test • Lestradet's test
Bilirubin	oxidation of bilirubin to green biliverdin or blue bilicyanin	• Rosin's test (oxidation of bilirubin with iodine) • Gmelin's test (with concentrated HNO_3)
Urobilinogen	reaction of urobilinogen with 4-dimethylaminobenzaldehyde in acidic medium producing a colored condensation product	• Ehrlich's test

2.2. Test strips

The test strips (diagnostic strips) enable detection of pathological urine components directly at patient's bed or in physician's consulting room. The strips consist of a plastic support onto which one or more indication zones are attached. In manufacture of the indication zones, a liquid analytic reagent is applied on a suitable absorbent (e.g. special filtration paper), and then gently dried. In Czech Republic the strips PHAN made by Lachema/Pliva a.s. are the most widely used.

There are various types of the diagnostic strips: they come either as monofunctional, or polyfunctional, or strips for specialised examinations.

Monofunctional strips contain basic indication zones for semiquantitative estimation of one urine component. **Polyfunctional strips** bear several indication zones and hence enable examination of several biochemical parameters simultaneously. They are designed for situations where it is useful to obtain as much information on the patient's health condition as possible, such as in various screening examinations.

Strips for specialized examinations include two or more indication zones chosen with respect to screening for or examination of a specific disease. For example strips for diabetes mellitus screening contain zones for ketone bodies and glucose, strips for renal diseases have zones for blood, protein, pH and nitrite, and strips for hepatic diseases examine bilirubin and urobilinogen.
The strips enable estimation of the following parameters in urine:

- protein
- glucose
- ketone bodies
- bilirubin
- urobilinogen
- hemoglobin, red blood cells
- ascorbic acid
- leukocytes
- nitrites
- pH
- specific gravity

The reactions involved in test strips detection are largely based on similar principles as the classical test-tube ones (table 1, table 2). Further we discuss in detail only the reactions not introduced in the previous practical lessons.

Ascorbic acid

Ascorbic acid (vitamin C) is examined because of its strong reducing properties that affect estimation of other analytes in urine. In particular, it interferes with reactions employing hydrogen peroxide, which reduces directly; and it also decomposes diazonium salts.

The principle of ascorbic acid detection utilises phosphomolybdenic acid, which is reduced with ascorbic acid to molybdenum blue. The test is not specific for ascorbic acid as other strong reducing agents would react in a similar manner.

Leukocytes

Chemical detection of leukocytes with the diagnostic strip is based on demonstration of enzymes **esterases** present in granulocytes. The esterase hydrolyses ester of indoxylcarbonic acid to indoxyl, which reacts with a stable **diazonium salt** yielding the corresponding **azo dye** (Fig. 1). In normal (negative) reaction the strip zone is colored creamy yellow; in case of positive reaction it turns pink or violet. The chemical demonstration of leukocytes cannot substitute the microscopic examination of urinary sediment. On the other hand, however, the biochemical examination can also find lysed leukocytes (e.g. in hypotonic urine), which is not possible with microscopy.

Leukocyturia comes generally as a sign of kidney or urinary tract inflammation. Majority of positive findings are caused by a bacterial infection of the urinary tract. In case of a positive leukocyte test it is recommended to perform also examination of proteinuria, hematuria, nitrituria, examination of the urinary sediment and microbiological examination.

Fig 1: Principle of detection of leukocytes in urine with the test strips:
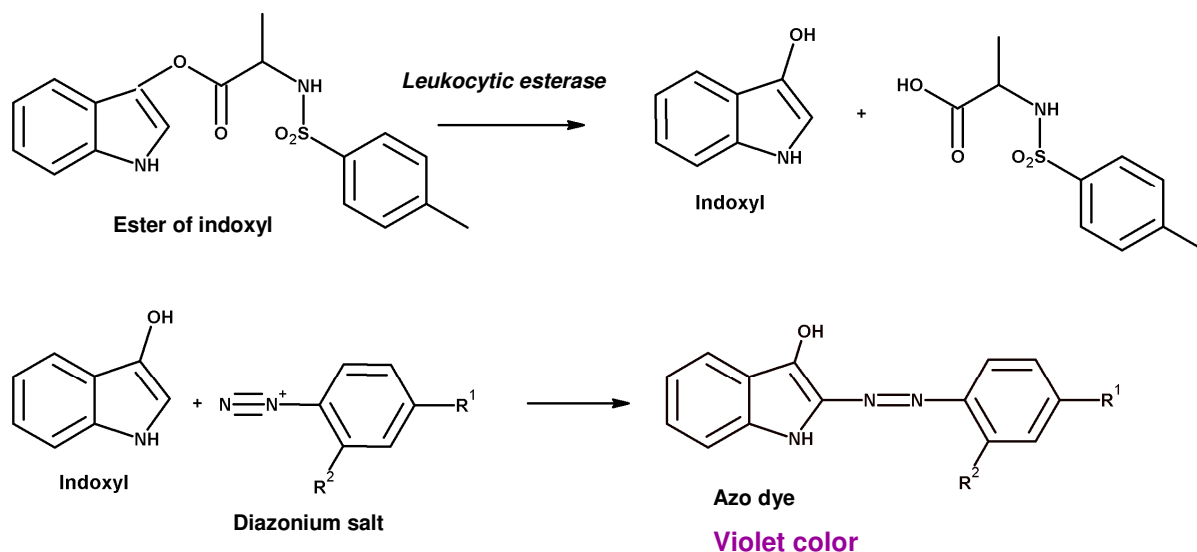
Nitrite (indirect detection of bacteria)

Normal urine does not contain nitrites. Some, in particular gram-negative bacteria such as Escherichia coli, Proteus, Klebsiella, staphylococci and others possess an ability to **reduce nitrates normally present in urine to nitrites**. The strips for indirect detection of bacteriuria use nitrites for the **Griess reaction**. Its principle is diazotation of sulfanilic acid with nitrites, originating in urine by bacterial reduction of nitrates, producing a diazonium salt. Azo-coupling follows that yielding a pink or even violet color (Fig. 2).

The test for nitrites in urine is necessary to perform with the early morning urine, because only in this case the stay of urine in the urinary bladder is long enough for bacterial reduction of nitrates to nitrites. Another recommendation is consumption of vegetables (contain nitrates) on the day before examination. *Positive detection of urinary nitrite confirms bacteriuria, while negative test does not exclude it.*

The indirect evidence of bacteriuria is only preliminary and does not replace microbiological examination.

Fig 2: Principle of detection of nitrites in urine with the test strips:
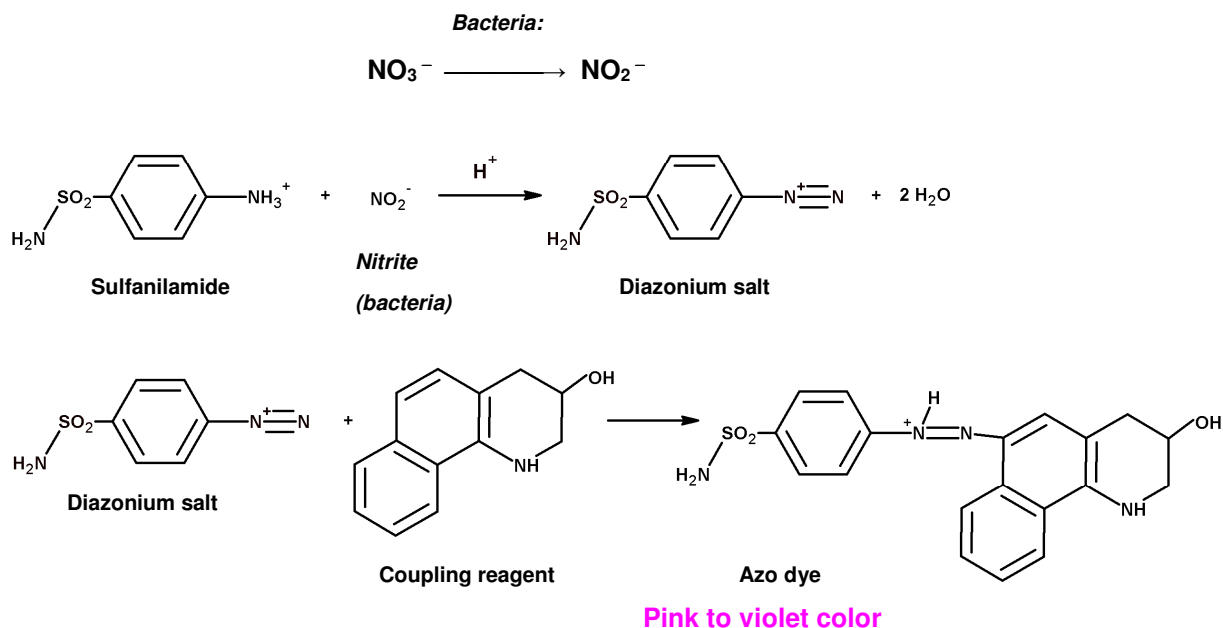
Table 2: Principles of detection of pathological components in urine with the test strips, and common causes of interference

Analyte	Principle	False positive results	False negative results
Protein	Protein error of acid-base indicator	Alkaline pH, contamination of sample vessel with disinfectants based on quaternary ammonium salts	Globulins and light chains of immunoglobulins are difficult to detect
Hemoglobin	Oxidation of chromogene with hydrogen peroxide catalyzed by pseudoperoxidase activity of hemoglobin	Microbial peroxidases, contamination of vessels with oxidizing disinfectants	High concentration of nitrites, vitamin C
Glucose	Enzyme method based on coupled glucose oxidase/ peroxidase reactions	Contamination of sample vessels with oxidizing disinfectants	Vitamin C, other reducing substances (homogentisic acid, DOPA), urinary infection
Ketone bodies	Reaction of acetoacetic acid and acetone with nitroprusside in alkaline medium (Legal test)	Substances with free sulfhydryl groups (e.g. captopril), some drugs based on phenolphthalein or sulfophthalein (laxancia) can stain due to alkaline reaction, phenylpyruvic acid (orange-red color)	β-hydroxybutyrate does not react
Bilirubin	Azo coupling of bilirubin with diazonium salt in acidic medium producing colored compounds	Exogenous substances in urine that have red color or turn red in strong acid	High contents of nitrites, light exposition
Urobilinogen	Azo coupling of urobilinogen with diazonium salt in acidic medium producing colored compounds	Exogenous substances in urine that have red color or turn red in strong acid	Formaldehyde, light exposition, old urine (oxidation to urobilin)
Nitrite	Griess reaction – diazotation of sulfanilamide with nitrite followed by azo coupling	Bacterial contamination	Lack of vegetables (nitrates) in diet, parenteral nutrition, Gram-positive bacteria, short stay of urine in urinary bladder, vitamin C
Leukocytes	Esterase activity of granulocytes and macrophages	Formaldehyde, alkaline pH, high specific gravity of urine	Vitamin C, some drugs

Procedure for urine examination with test strips:

Take out only the strips that are to be used immediately, and close the tube with remaining strips again. Never touch the strip indication zones with bare hand. The strips should be stored in original containers, tightly closed with a desiccant, in a dark and dry place at temperature +2 to +30 ˚C. When examining a urine sample, dip the strip into the urine so that all the zones are immersed for 2–3 seconds. Then take the strip out and wipe the excess of urine by touching the walls of sample container. Keep the strip in a horizontal position to avoid mixing of reagents from different reaction zones. Wait for the recommended incubation time (usually 60 seconds, for leukocytes 120 seconds), and evaluate the resulting color changes. The evaluation is possible either:
- *Subjectively by comparison with the color scale on the tube label*.
- *Objectively by means of reflection photometers*, which measure the intensity of light reflected from the strip reaction zone.

3. Simple chemical reactions in urine used in screening of inborn errors of metabolism

Biochemical investigation of inborn errors of metabolism of amino acids includes several steps. It usually starts with **screening**. Screening investigations consist of simple tests performed with blood or urine samples and are based on various principles; some of them are carried out at departments of obstetrics on all the babies newly born in the department.

A good screening test should typically have negligible low chance of false negative result, but may quite often be false positive. When positivity is found in any screening reaction, it does not yet make diagnosis, but rather it leads to further more detailed examinations.

Screening tests for amino acid inborn errors include:
1. Simple chemical reactions in urine
2. Chromatographic methods
3. Indicator papers
4. Bacterial inhibition tests

In some inborn errors urine has a typical smell caused by the abnormal metabolite accumulation.

Selected simple chemical reactions in urine:

Fölling′s test with ferric chloride

Reaction with ferric chloride is a classical test for the screening detection of newborn with **phenylketonuria**. Ferric chloride gives with phenylpyruvate present in the patient urine a green complex. However, other compounds also react with Fe^{3+} producing complexes of various colors (Table 3).

Hyperphenylalaninemia (phenylketonuria) is a classical example of an inborn error of amino acids and also one of the most important in medical practice. Phenylketonuria is a disease caused by a total defect of hydroxylation of phenylalanine to tyrosine that is normally catalyzed by phenylalanine-4-monooxygenase (phenylalanine-4-hydroxylase) in the presence of oxygen and tetrahydrobiopterin as a coenzyme. Phenylalanine accumulates in the blood. Urinary excretion of this substance as well as of its pathologic metabolites (phenylpyruvate, phenyllactate and phenylacetate) increases. Phenylpyruvate can be demonstrated by the reaction with $FeCl_3$ (the Fölling's test). Unless recognized early after birth, the phenylketonuria results in severe and irreversible brain damage and mental retardation. On the other hand, early restriction of phenylalanine in the diet can totally prevent the damage and the child can develop normally.

Brand's test with sodium nitroprusside

Reaction of sodium nitroprusside with **sulfhydryl compounds** (e.g. cysteine, homocysteine) yields rose or purple-red complex products:

$$[Fe^{II}(CN)_5NO]^{2-} + S^{2-} \rightarrow [Fe^{II}(CN)_5NOS]^{4-}$$
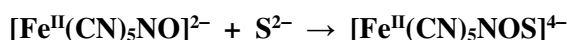
Disulfides (cystine, homocystine) do not give the reaction and therefore must be first reduced to free sulfhydryls by alkaline sodium cyanide.

Cystinuria is one of inborn errors caused by a disorder in the transport of amino acids in the kidney and gut. It is caused by a defect in the reverse transport of cystine, lysine, arginine and ornithine in the epithelial cells of renal tubuli and digestive tract. Biochemical tests reveal increased urinary excretion of all the mentioned amino acids. Cystine urolithiasis is the major clinical sign; it is caused by a low water solubility of cystine. The presence of cystine in urine can be demonstrated by the Brand's test with sodium nitroprusside.

Dinitrophenylhydrazine test for ketoaciduria

2,4-dinitrophenylhydrazine in acidic medium reacts with 2-oxo acids (α-keto acids) and forms precipitates of hydrazones. Positive test indicates elevated excretion of keto acids or other ketone bodies in urine.
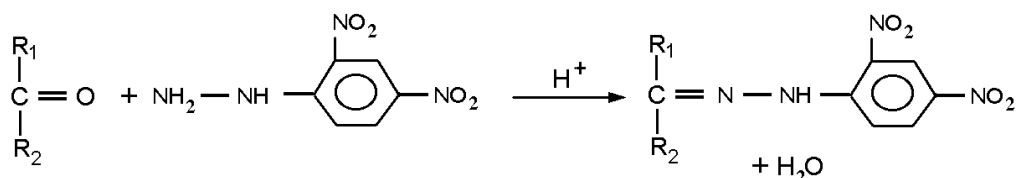
Leucinosis (maple syrup urine disease) develops due to defect in the enzyme system performing oxidative decarboxylation of branched-chain α-ketoacids. High levels of leucine, valine and isoleucine are found in the serum. Oxo-acids that are formed by transamination of these amino acids (2-oxoisovalerate, 2-oxo-3-methylvalerate and 2-oxoisocapronate) are excreted in urine in amounts 10-100 higher than normal. The oxo-acids in urine can be demonstrated by the reaction with 2,4-dinitrophenylhydrazine.

Table 3: Examples of screenings reactions in urine

Test	*Reacting substance*	*Color*
Fölling's test	phenylpyruvate	permanent bluish-green
	p-hydroxyphenylpyruvate	transient green
	branched chain keto acids	greyish-blue
	salicylates	purple
	homogentisate	transient blue
Brand's test	cysteine	red
	homocysteine	red
	disulfide of cysteine & homocysteine	red
2,4-Dinitrophenylhydrazine	branched chain keto acids	brownish-wine
	phenylpyruvate	brownish-wine
	p-hydroxyphenylpyruvate	brownish-wine