THE LABORATORY ASPECTS OF PROTEINURIA

Velibor ČABARKAPA 1,2, Mirjana DERIĆ 1,2, Branislava ILINČIĆ 1,2, Biljana VUČKOVIĆ 1,2, Aleksandra TRIFU 3 and Mirko ŠIPOVAC 3

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Summary
Introduction. The existence of proteinuria may be overlooked by applying the test strips. The aim of this study has been to determine the discrepancy between the findings of proteinuria detected by test strips when compared to the results of its testing with the sulfosalicylic acid. Material and Methods. The study sample consisted of 1,106 subjects, who were divided into the proteinuria positive (test strips showed the presence of isolated proteinuria), and proteinuria negative group (microscopic examination revealed the presence of ≥10 fresh red blood cells/µL, and/or ≥1 dysmorphic erythrocyte/µL, and/or ≥10 leukocytes/µL, and/or ≥1 cylinder, and/or ≥1 nonsquamous epithelial cells/µL, and/or ≥100 bacteria/µL). Both groups had the urine tested with sulfosalicylic acid. The chemical and microscopic examination of the urine was done by the analyzer LabUMat-UriSed.

Results. Proteinuria was confirmed with the sulfosalicylic acid test in 96.5% of subjects from group 1 and in 85.3% of subjects from group 2. Among the patients with the negative finding of proteinuria on the test strip and with the positive sulfosalicylic acid test there was a significantly higher number of those with pathological findings of erythrocytes, leukocytes, bacteria and cylinders in the urine when compared to those of the same group with negative sulfosalicylic acid test. Conclusion. Sulfosalicylic acid test should be performed in cases of pathological microscopic findings in the urine in case of the presence of ≥10 fresh erythrocytes/µL and/or ≥1 dysmorphic erythrocyte/µL and/or ≥10 leukocytes/µL and/or ≥1 cylinder (except hyaline) and/or ≥1 nonsquamous epithelial cells/µL and/or ≥100 bacteria/µL even if the test strip examination is negative for proteinuria.

Keywords: Proteinuria; Reagent Strips; Urinalysis; Salicylates; Morphological and Microscopic Findings; Urine Specimen Collection

Sažetak
Uvod. Postojanje proteinurije se može prevideti upotrebom test-trake. Cilj ove studije je da se utvrdi diskrepanca između nalaza proteinurije otkrivene test-trakama u odnosu na rezultate testiranja sa sulfosaliloksim kiselinom. Materiaj i metode. Ucupno 1 106 ispitanika, podeljeno je u grupu pozitivnih na proteinuriju (prisustvo izolovane proteinurije na test-traci), kao i i na grupu negativnih na proteinuriju (mikroskopski pregled otkriva prisustvo ≥10 svežih eritrocita/µl , i/ili ≥1 dismoričnih eritrocita/µl, i/ili ≥100 bakterija/µl). Obe grupe, urin je testiran sulfosaliloksim kiselinom. Hemijski i mikroskopski pregled urina vršen je na analizatoru LabUMat.

Rezultati. Testom sulfosaliloksim kiselinom proteinurij na je potvrđena kod 96.5% ispitanika prve grupe, kao i kod 85,3% ispitanika druge grupe. Kod ispitanika sa negativnim nalazom proteinurije na test-traci i sa pozitivnim testom sa sulfosaliloksim kiselinom postoji znatno veći broj onih sa patološkim nalazima eritrocita, leukocita, bakterija i cilindara u urinu u odnosu na ispitanike iste grupe sa negativnim testom sa sulfosaliloksim kiselinom. Zaključak. Testiranje sulfosaliloksim kiselinom trebalo bi primenjivati u slučaju patološkog mikroskopskog nalaza u urinu ukoliko postoji prisustvo ≥10 svežih eritrocita/µl i/ili ≥1 dismoričnih eritrocita/µl i/ili ≥100 bakterija/µl. U obe grupe, urin je testiran sulfosaliloksim kiselinom. Hemijski i mikroskopski pregled urina vršen je na analizatoru LabUMat.

Ključne reči: proteinurija; test trake; analiza urina; salicilati; morfološki i mikroskopski nalazi; sakupljanje uzorka urina

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Corresponding Author: Doc. dr Velibor Čabarkapa, Klinički centar Vojvodine, Centar za laboratorijsku medicinu, 210000 Novi Sad, Hajduk Veljkova 1-7, E-mail: veliborcabarkapa@gmail.com
morning urine, usually as a part of a systematic examination or during health check-ups for employees [1]. The presence of hematuria and/or proteinuria in the absence of any obvious impairment of the kidney function as well as any symptom or sign of kidney disease is referred to as asymptomatic urinary abnormalities (AUA) [2]. To confirm AUA it is necessary to repeat the examination of the first morning urine and to perform additional diagnostic procedures in order to detect kidney disease. It is of great importance to identify this category of patients so as to provide them the prompt therapy and thus delay the progression of the disease.

However, praxis shows that both doctors and patients rarely pay full attention to AUA. This is partly due to certain errors that occur before performing the laboratory tests (pre analytical phase) such as improper collection of the urine and un timely delivering of the urine samples to the laboratory, and errors that are related to the phase of the analysis (the analytical phase) due to the presence of various interfering factors, some analytical limitations, etc. A good part of these errors can be contributed to the inadequate cooperation between patients and doctors.

Proteinuria can be a harmless finding if it is detected in only one urine sample, while persistent proteinuria may indicate the presence of a kidney disease. Proteinuria associated with erythrocyturia indicates glomerulopathy while proteinuria associated with leukocyturia, bacteriuria and non-squamous epithelial cells (NEC) indicates a tubulointerstitial disease. In addition, albuminuria as a marker of kidney impairment is a parameter for diagnosing the first stage of chronic kidney disease (CKD), even in terms of a preserved glomerular filtration rate (GFR) [3]. Besides, persistent albuminuria is a characteristic finding for initial diabetic nephropathy.

The standard method to detect proteinuria is chemical testing of the first morning urine using test strips. However, most test strips, even of different manufacturers, are mainly based on the principle of detecting albumin, and therefore the glomerular type of proteinuria. In addition, test strips can give false positive results such as in alkaline urine, highly concentrated urine, hematuria, when using certain drugs, etc., or false negative results such as in diluted urine (relative density <1.015) or when the urinary proteins are of non-albumin nature [4]. Indirect evidence of proteinuria is the presence of cylinders in the microscopic examination of urine, while the chemical test with sulfosalicylic acid (SSA) is a confirming test for the presence of proteinuria.

Since it is assumed that the existence of proteinuria may be overlooked by applying test strips, the aim of this study has been to determine the discrepancy between the findings of proteinuria detected by chemical examination of urine by test strips when compared to the results of its testing with the SSA. Moreover, in a number of samples with the mentioned discrepancy, quantitative tests were used to determine the degree of proteinuria and to determine the predominant type of proteinuria.

**Material and Methods**

This study was conducted at the Clinical Center of Vojvodina (CCV), Novi Sad by reviewing the medical records of the routine examination of the first morning urine in the period from October 2014 to February 2015. Out of 6835 examined patients (aged over 18 years), 1106 subjects were included in the study based on the finding of proteinuria which was detected by the chemical examination of urine using test strips and by the finding of the microscopic examination of the urine. The subjects were divided into two groups. Group 1 (proteinuria positive respondents) consisted of patients in whom the test strips showed the presence of isolated proteinuria (proteinuria positive results on the test strips with a normal chemical and microscopic examination of urine). Group 2 (proteinuria negative subjects) consisted of subjects whose chemical examination done by means of test strips was negative for the presence of proteins but the microscopic examination revealed the presence of >10 fresh red blood cells/µL (reference interval 0-5/µL) and/or ≥1 dysmorphic erythrocyte/µL (reference interval 0-0/µL) and/or ≥10 leucocytes/µL (reference interval 0-5/µL) and/or ≥1 cylinder (except hyaline) (reference range 0-0/µL, and for the hyaline cylinders the reference interval 0-1/µL) and/or ≥1 NEC/µL (reference interval 0-3/µL), and/or ≥100 bacteria/µL (reference interval for the bacteria is 0-20/µL).

The urine of patients from both groups was tested with SSA; in group 1 it was used as a confirming test for the presence of isolated proteinuria, and in the other group as a complementary diagnostic test for detecting proteinuria. The test was performed by adding 5-10 drops of 20% SSA in about 5 ml of urine. Depending on the quantity of proteins present in the urine, cloudiness i.e. turbidity of some degree occurs. SSA test result was expressed either as "negative reaction with SSA" (corresponding to <0.1 g/L) or if it was positive: barely noticeable turbidity (corresponding to about 0.2 g/L), 1+ (corresponding to 0.3-1 g/L), 2+ (corresponding to 1.2-5 g/L), 3+ (corresponding to 2.5-4.5 g/L), or 4+ (corresponding to >4.5 g/L). Result reading of the SSA test was performed by a single laboratory technician with years of experience.

**Abbreviations**

- A/C – albumin/creatinine ratio
- AUA – asymptomatic urinary abnormalities
- CKD – chronic kidney disease
- CCV – Clinical Center of Vojvodina
- GFR – glomerular filtration rate
- ESRD – end stage renal disease
- NEC – nonsquamous epithelial cells
- P/C – protein/creatinine ratio
- SDS – Na-dodecyl sulfate
- SSA – sulfosalicylic acid
- AUA – asymptomatic urinary abnormalities
- A/C – albumin/creatinine ratio
- SSA – sulfosalicylic acid
Table 1. The results of the chemical examination of urine using test strips for detecting proteinuria and the sulfosalicylic acid test (SSA) in all subjects

<table>
<thead>
<tr>
<th>Proteinuria – chemical examination of urine via test strips</th>
<th>N</th>
<th>SSA+ (n (%)</th>
<th>SSA- (n (%))</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative/Negativno</td>
<td>962</td>
<td>821 (85.3%)</td>
<td>141 (14.7%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Positive/Pozitivno</td>
<td>144</td>
<td>139 (96.5%)</td>
<td>5 (3.5%)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Legend: SSA+: positive sulfosalicylic acid test; SSA-: negative sulfosalicylic acid test; N (n) - the number of subjects

All subjects underwent chemical and microscopic examination of the first morning urine by the automated analyzer LabUMat-UriSed (77 ElektronikaKft., Hungary) at the Center for Laboratory Medicine of CCV. The chemical examination of urine was done with LabStrip U11 Plus test strips (77 ElektronikaKft., Hungary) as each strip has 11 test fields with reagents for the determination of 11 standard parameters for the chemical examination of urine. The microscopic examination of the urine was performed by the flow cytometry method after the chemical examination, without prior centrifugation of the sample.

In 29 patients in whom the chemical examination using test strips was negative for proteinuria but the SSA test was positive, quantitative tests were performed to measure the concentration of proteins, albumin and creatinine, and to determine the type of proteinuria in the same sample of the first morning urine. The following ratios were also calculated: the protein/creatinine (P/C) ratio (mg/mmol), and the albumin/creatinine ratio (A/C) (mg/mmol). The P/C ratio above 22 mg/mmol and the A/C ratio above 3.0 mg/mmol were considered to be a pathological finding [3, 5]. The concentration of protein in the urine was determined by the turbidimetric method with benzethonium chloride, and the level of creatinine was determined by the Jaffe method with alkaline picrate, all by means of commercial kits (Abbott, Wiesbaden, Germany) on the biochemical analyzer ci4000 Architect. The concentration of albumin in the urine was determined by the immunoturbidimetric biochemical analyzer ADVIA 1800 with commercial kits (Siemens Healthcare Diagnostics, Germany). The type of proteinuria was determined using commercial agarose gels Hydragel 5 Proteinurie (Sebia, France). After adding the anionic detergent (Na-dodecyl sulfate (SDS)) proteins form complexes with SDS hence disrupting the native conformation of the proteins and a new one is established with the same conformation and the same negative charge per mass unit. Proteins were then separated by electrophoresis according to their molecular weights.

Statistical analysis was performed by means of STATISTICA 12.0 statistical software (StatSoft, Inc., Tulsa, OK, USA), for which the University of Novi Sad has the university license. The results were presented in tables and graphs. Chi-square test showed the differences between individual variables. A p value of <0.05 was considered statistically significant.

Results

The study included 1106 patients, 507 men and 599 women, their age being 55.3 ± 17.1 years.

Isolated proteinuria (positive finding on the test strip with a normal remaining chemical and microscopic examination of urine) was detected in 144 subject (13%) and proteinuria on the test strip was negative in the remaining 962 persons (87%) despite the pathological microscopic findings (the presence of ≥10 fresh erythrocytes/µL and/or ≥1 dysmorphic erythrocyte/µL and/or ≥10 leukocytes/µL and/or ≥1 cylinder (except hyaline) and/or ≥1 NEC/µL and/or ≥100 bacteria/µL).

Proteinuria was confirmed with the SSA test in 96.5% of the proteinuria positive subjects from group 1 (Table 1). The SSA test was positive in 85.3% of proteinuria negative subjects, which is significantly higher in relation to the negative SSA testing (p <0.001).

Among the patients with the negative finding of proteinuria on the test strip and with the positive SSA test there is a significantly higher number of those with pathological findings of erythrocytes in the urine when compared to those of the same group with negative SSA test, in the form of an increased number of fresh erythrocytes and/or the presence of dysmorphic erythrocytes, as well as the isolated presence of dysmorphic erythrocytes (p <0.05) (Graph 1).

Among the subjects with negative findings of proteinuria on the test strip examination but with positive findings for it when tested with SSA there was a significantly higher number of those with pathological findings of leukocytes (>10/µL), bacteria (>100/µL) and cylinders (>1/µL) in the urine when compared to the same group of subjects with the negative test with SSA (p <0.05), while the number of patients with NEC>1/µL was significantly higher among those with the negative SSA test (Graph 2).

The specific gravity of the urine ≥1.015 was present in almost half of all subjects (46.1%) with negative findings of proteinuria on the test strip examination and with positive findings on the SSA test (Graph 3).

In the subgroup of 29 patients with negative proteinuria on the test strip examination and positive SSA test, it was observed that an abnormal A/C ratio accompanied with pathological P/C ratio was present...
in 15 subjects (51.7%), abnormal A/C ratio with the normal P/C ratio was present in 2 subjects (6.9%), while A/C and the P/C ratio was within the limits of the reference range in 12 subjects. Tubular type of proteinuria was present in 14 subjects; mixed type in only 1 subject, the findings pointed to the presence of microalbuminuria in 2 subjects and they were negative for proteinuria on the agarose gels test in 12 subjects (Figure 1).

Graph 1. The frequency (%) of the pathological number of erythrocytes in the urine

Legend: SSA+: positive sulfosalicylic acid test; SSA–: negative sulfosalicylic acid test

**Discussion**

Proteinuria represents protein excretion in the urine in a quantity exceeding 150 mg/day [6]. This occurrence is benign, i.e. it is a harmless finding when it is caused by dehydration, fever, severe emotional stress, inflammatory processes of the genitourinary tract (of a non-kidney origin), intensive physical activity, postural mechanisms, or due to exposure to low temperature [7]. Therefore, if the results of the examination of the first morning urine are abnormal, it must be a routine to examine new urine samples after the cessation of conditions that may lead to benign proteinuria.

However, persistent proteinuria indicates the presence of a renal disease, such as glomerular diseases
as a part of the primary and secondary glomerulopathies, and tubular diseases as a part of the tubulointerstitial nephropathies such as those caused by the use of some drugs (non-steroidal anti-inflammatory drugs, antibiotics, etc.), pyelonephritis, hypokalemia, hypercalcemia and others. In addition, proteinuria is one of the most important predictors of CKD progression [8, 9]. Increased and persistent protein reabsorption in tubulocytes leads to the damage of these cells and impairs their function [10].

The first step in the detection of proteinuria is the chemical analysis of the urine using test strips, which is based on the “protein error” of the pH indicators, or the capability of proteins to change the color of some acid-base indicators without changing the pH [4]. The sensitivity of the test strip for protein depends on the manufacturer and it ranges from 0.1-0.3 g/L [4]. The test strips are usually sensitive to the glomerular type of proteinuria because they are sensitive to the presence of albumin [11], while they are insensitive to proteins of non-albumin nature, which could be found in urine such as the light chains of immunoglobulins. There are numerous interfering factors that can lead to false positive and false negative results.

Test strips may give false positive results as it can be in case of alkaline urine (pH > 7.5), if the test strip is held submerged in urine for too long, in highly concentrated urine, intense hematuria, when using penicillin, sulfonamides and other drugs, in the presence of pus, seed or vaginal secretions. False-negative results occur in dilute urine (specific gravity <1.015) or when the urinary proteins are of non-albumin nature, i.e. of low molecular weight [4, 12].

If the test strip indicates the presence of protein in the urine, it is recommended to do one of the confirmatory tests [4, 13], which implies that the confirmatory test has to have at least the same sensitivity and better specificity than the primary test. In laboratory practice, the preference is to use the SSA test, which is based on the chemical precipitation of all proteins [14]. The sensitivity of the SSA test is 0.1 g/L. However, with the use of the SSA it is possible to obtain false-positive results (use of contrast agents in radiology, high concentrations of antibiotics - penicillin, cephalosporins, present turbidity in the urine, which is why the SSA test is performed in centrifuged urine) and false negative results (highly buffered alkaline urine which neutralizes the SSA reagent, diluted urine, turbid urine) [15].

In this study, proteinuria was confirmed with the SSA test in 96.5% of proteinuria positive subjects from group 1, and in only 5 (3.5%) the SSA test was negative, which may be due to false negative SSA test results, or false positive test strip results.

However, proteinuria may be present despite the negative test strip results [16]. Proteinuria is a frequent companion of hematuria in glomerular kidney diseases, leukocyturia and bacteriuria in inflammatory diseases of the kidney and some tubulopathies. In addition, the presence of proteins in the urine is the starting point for creating cylinders [6]. Therefore, we have established criteria to perform the SSA test when the test strip examination was negative in those subjects who had pathological findings in the microscopic examination of the urine. The criteria applied in this study relate to the presence of >10 fresh erythrocytes/µL and/or ≥1 dysmorphic erythrocyte/µL and/or ≥10 leukocytes/µL and/or ≥1 cylinder (except hyaline) and/or ≥1 NEC/µL and/or ≥100 bacteria/µL. We have found that the SSA test was positive in 85.3% of the subjects, which is significantly higher than the number of subjects with a negative SSA test (p < 0.001). Besides, a significant number of these subjects had erythrocyturia (>70%) and leukocyturia (>70%), which was also significantly higher than in the group of the subjects with negative SSA test (p < 0.001). In addition, cylindruria was present in a significant number of these subjects when compared to the number of those with negative SSA test (p < 0.05), as opposed to the pathological findings NEC which were more frequent in subjects with negative SSA test (p < 0.05). False negative results of proteinuria obtained by chemical testing using test strips may be due to diluted urine (relative density <1.015). In these subjects (with false negative proteinuria) 56% of the samples had a relative density less than 1.015 which is most likely the most common cause of the discrepancy between the chemical examination of urine by test strips when compared to the use of the SSA test.

Another cause of false negative results of proteinuria when using test strips is the presence of proteins of non-albumin nature, i.e. low molecular weight proteins. After confirming the presence of pathological proteinuria via a confirmative test such as the SSA test, it is necessary to determine the PC and/or A/C ratio in the first morning urine sample or the excretion of protein/albumin in the 24 hour collected urine sample and possibly determine the type of proteinuria with electrophoretic techniques [17, 18]. In a group of 29 such subjects (with test strip negative and SSA positive proteinuria), 15 subjects had abnormal PC ratio, while in most, tubular type of proteinuria was confirmed using electrophoretic techniques.

**Conclusion**

Examination of the first morning urine should be carried out by automated analyzers because centrifuging of the sample is avoided after being chemically examined by means of test strips, thus avoiding the loss of certain elements in the urine. Elements that indicate kidney damage in the microscopic examination of urine are kidney tubular cells, erythrocytes, leukocytes, granulated, wide cylinders and dysmorphic erythrocytes [19]. However, it should be kept in mind that potential errors may occur mainly due to poor cooperation between clinicians and patients. Clinicians should give clear instructions on how to collect a urine sample correctly, verify the patient’s compliance, and must properly take the anamnesis related to all potentially interfering factors. Another thing that
should be kept in mind is that the sensitivity of the test strips is not very high, that it depends on the manufacturer, and that it is mainly based on the determination of albumin. Therefore, we believe that the confirmatory sulfosalicylic acid test should be performed in all cases of pathological microscopic findings in the urine (presence of \( \geq 10 \) fresh erythrocytes/\( \mu L \) and/or \( \geq 1 \) dysmorphic erythrocyte/\( \mu L \) and/or \( \geq 10 \) leukocytes/\( \mu L \) and/or \( \geq 1 \) cylinder (except hyaline) and/or \( \geq 1 \) nonsquamous epithelial cells/\( \mu L \) and/or \( \geq 100 \) bacteria/\( \mu L \)) even if the test strip examination is negative for proteinuria.

References


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