

### Evaluation of the appropriate time period between sampling and analyzing for automated urinalysis

Ramona C. Dolscheid-Pommerich<sup>\*1</sup>, Ute Klarmann-Schulz<sup>2,3</sup>, Rupert Conrad<sup>4</sup>, Birgit Stoffel-Wagner<sup>1</sup>, Berndt Zur<sup>1</sup>

<sup>1</sup>Department of Clinical Chemistry and Clinical Pharmacology, University Hospital Bonn, Bonn, Germany

<sup>2</sup>Institute of Medical Microbiology, Immunology and Parasitology, University Hospital Bonn, Bonn, Germany

<sup>3</sup>Institute of Medical Biometry, Informatics and Epidemiology (IMBIE), University Hospital Bonn, Bonn, Germany

<sup>4</sup>Department of Psychosomatic Medicine and Psychotherapy, University Hospital Bonn, Bonn, Germany

Corresponding author: ramona.dolscheid-pommerich@ukb.uni-bonn.de

#### Abstract

**Introduction:** Preanalytical specifications for urinalysis must be strictly adhered to avoid false interpretations. Aim of the present study is to examine whether the preanalytical factor 'time point of analysis' significantly influences stability of urine samples for urine particle and dipstick analysis.

**Materials and methods:** In 321 pathological spontaneous urine samples, urine dipstick (Urisys™2400, Combur-10-Test™strips, Roche Diagnostics, Mannheim, Germany) and particle analysis (UF-1000 i™, Sysmex, Norderstedt, Germany) were performed within 90 min, 120 min and 240 min after urine collection.

**Results:** For urine particle analysis, a significant increase in conductivity (120 vs. 90 min:  $P < 0.001$ , 240 vs. 90 min:  $P < 0.001$ ) and a significant decrease in WBC (120 vs. 90 min  $P < 0.001$ , 240 vs. 90 min  $P < 0.001$ ), RBC (120 vs. 90 min  $P < 0.001$ , 240 vs. 90 min  $P < 0.001$ ), casts (120 vs. 90 min  $P < 0.001$ , 240 vs. 90 min  $P < 0.001$ ) and epithelial cells (120 vs. 90 min  $P = 0.610$ , 240 vs. 90 min  $P = 0.041$ ) were found. There were no significant changes for bacteria. Regarding urine dipstick analysis, misclassification rates between measurements were significant for pH (120 vs. 90 min  $P < 0.001$ , 240 vs. 90 min  $P < 0.001$ ), leukocytes (120 vs. 90 min  $P < 0.001$ , 240 vs. 90 min  $P < 0.001$ ), nitrite (120 vs. 90 min  $P < 0.001$ , 240 vs. 90 min  $P < 0.001$ ), protein (120 vs. 90 min  $P < 0.001$ , 240 vs. 90 min  $P < 0.001$ ), ketone (120 vs. 90 min  $P < 0.001$ , 240 vs. 90 min  $P < 0.001$ ), blood (120 vs. 90 min  $P < 0.001$ , 240 vs. 90 min  $P < 0.001$ ), specific gravity (120 vs. 90 min  $P < 0.001$ , 240 vs. 90 min  $P < 0.001$ ) and urobilinogen (120 vs. 90 min,  $P = 0.031$ ). Misclassification rates were not significant for glucose and bilirubin.

**Conclusion:** Most parameters critically depend on the time window between sampling and analysis. Our study stresses the importance of adherence to early time points in urinalysis (within 90 min).

**Key words:** urinalysis; automation; analytical sample preparation methods; flow cytometry; specimen handling

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#### Introduction

Urinalysis with urine dipsticks and urine particle analysis are established methods in the daily routine of diagnosis and control of urinary tract infections (UTI) in hospital laboratories as well as outpatient departments. Various types of indication for urinalysis exist, including diagnosis, disease monitoring, screening, check-up, and therapy control in infections, bleedings and diseases of the kidneys and the urinary tract, metabolic disorders (e.g. diabetes mellitus), liver and hematological diseases

(1). Aiming for continuous improvement in diagnosis, automated urinalysis was established and has now become part of standard examinations (2,3). Studies comparing urine particle counts by urine flow cytometry using UF 1000i™ (Sysmex, Norderstedt, Germany) and bacterial culture showed a good correlation and in part a better performance (4,5). However, the current stage of objectivity and standardization cannot prevent potential sources of error in preanalytics. In view of the fact that UTIs

are among the most common infections worldwide, preanalytical errors can have a far-reaching impact (6). In the US, more than seven million cases of urinary tract infections are seen by physicians every year (7). The European Guidelines for Urinalysis list urine dipstick analysis and microscopy as alternative diagnostic tools in uncomplicated cystitis and recommend antibiotic therapy in line with an existing concomitant disease (8). Correct urinalysis results are especially important for distinguishing between physiological and pathological cell numbers. Further diagnostic approaches as well as any clinical decision for or against therapy are based on this analysis. Consequently, false results in urinalysis cause a high risk of incorrect over- or undertreatment.

The European Guidelines for Urinalysis were first published in 2000 (8). Since more than 50% of all faulty laboratory results are preanalytical, the urinalysis guidelines provide clear instructions for urinalysis, especially regarding time of analysis and correct adherence to standardized preanalytical procedures (9). To ensure correct analysis, the current version of the CLSI Guidelines from 2009 provides important information on different types of urine specimens, detailed descriptions regarding definition of first morning urine specimen and collection of clean catch specimens from male and female patients. Also, details on transport and storage in relation to urinalysis preanalytics are provided. Regarding specimen acceptability, the CLSI Guidelines recommend urinalysis within two hours from sampling. If this is not possible, samples should be stored immediately at 4 °C. Further analyses should be performed within four hours (1). Urine cultures can be analyzed at 4 °C for a period of 24 hours (h) (10). One study analyzing urine samples, which were stored for 2h and 4h at room temperature without addition of preservatives, found no significant differences when comparing the results (11). The reference ranges for urine parameters are problematic (12). Some threshold values reported in the literature are inconsistent. For example, for UF-100™ (Sysmex, Norderstedt, Germany) in a collective of 1005 samples, the cut-off value for leucocytes (WBC) was given as 111 WBC /  $\mu\text{L}$  and for bacteria, 3000 bacteria /  $\mu\text{L}$ . While for

WBC, this was within the reference range as recommended by the manufacturer, the cut-off value for bacteria as recommended by the manufacturer was in fact 8000 bacteria /  $\mu\text{L}$  (13). Another critical aspect in urinalysis is the time interval of sample analysis after collection. During sample storage, the preanalytical focus must not only be on correct storage, but also on sample stability (14).

It remains unclear, however, on what data the recommendations regarding time for analysis are based on. Limited information is available regarding behaviour and/or changes in urinalysis/measurement results from early analysis times (11). As this may have immediate consequences for the patients, it remains to be investigated whether there is in fact a critical time period within which urinalysis must be performed. Therefore, the aim of the present study was to examine whether the preanalytical factor 'time point of analysis' significantly influences stability of urine samples in urine particle analysis and urine dipstick analysis.

## Materials and methods

### Materials

In our study, we analyzed 321 pathological urine samples (spontaneous urine, mid stream, clean catch specimens) collected over a period of two months. Samples were collected in 10 mL Urine Monovettes (Sarstedt, Nümbrecht, Germany) without bacteria growth inhibitors and analyzed immediately after collection.

All samples were urine samples from the routine collective of the central laboratory at the University Clinics Bonn, Germany (collected at the wards and outpatient departments of the University Clinics Bonn, Germany) as part of clinical examinations. All samples had to fulfil one of the following inclusion criteria at the first measurement: erythrocytes > 20 /  $\mu\text{L}$ , leucocytes > 20 /  $\mu\text{L}$ , bacteria > 100 /  $\mu\text{L}$ , dipstick positive for blood/leucocytes/nitrite. Samples from pediatric patients were excluded. To assure the performance of the first analysis within 90 min, sample arrival was monitored in cooperation with the wards and outpatient departments' staff. Urine samples were stored in capped

containers at room temperature between analyses.

## Methods

Urine dipstick measurements were performed with Urisys™ 2400 (Roche Diagnostics GmbH, Mannheim, Germany) using Combur<sup>10</sup>Test™ strips. This analyzer uses the following measurement methods: reflectance photometry (wave lengths 470 nm, 555 nm, 620 nm; refractometry (density), turbidimetry (cloudiness)). Urine particle analysis was performed with UF-1000i™ (Sysmex, Norderstedt, Germany) as quantitative and semi-quantitative analysis. This instrument uses the following measurement methods: fluorescence flow cytometry with diode laser and hydrodynamic focusing and conductometry. The reference ranges were as recommended by the respective manufacturers.

Measurements were done within 90 min, 120 min and 240 min from sample collection. The measurement time point of 90 min was chosen since it represents a feasible preanalytical time period in our daily routine.

Prior to each measurement, the urine was mixed automatically as well as manually. First, urine dipstick analysis was performed for the following parameters: specific weight, pH, leucocytes, erythrocytes, nitrite, protein, glucose, ketone, urobilinogen, bilirubin. Next, automated urine particle analysis was performed for the following parameters: conductivity, white blood cell count (WBC), red blood cell count (RBC), epithelial cells (EC), bacteria (bac) and casts. For each measurement, 4 mL of urine were used.

## Statistical analysis

Data were statistically analyzed using the IBM SPSS Statistics, Version 20 (IBM Corporation, New York, United States). The Kolmogorov-Smirnov, Friedman test and McNemar's tests for paired samples were used.  $P < 0.05$  was considered statistically significant: Variables of urine particle analysis were described by median and interquartile range (IQR). For urine dipstick parameters, misclassification rates were analyzed. Normality of distribution was

tested by Kolmogorov-Smirnov tests for all variables. All parameters were non-normally distributed. Continuous data were tested by nonparametric tests for repeated measures (Friedman tests). Categorical data were examined by McNemar's tests for paired samples. Categorical data in categories where frequencies were low ( $< 5$ ) were merged to categories with frequencies of at least ten.

## Results

### Urine particle analysis

The particle analysis parameters investigated are shown in Table 1. A significant increase in conductivity at 120 min and 240 min after urine collection was noted (conductivity 120 vs. 90 min:  $P < 0.001$ , 240 vs. 90 min:  $P < 0.001$ ). Regarding WBC, RBC and casts, a significant decrease in cell numbers was found at 120 min and 240 min after urine collection (WBC 120 vs. 90 min  $P < 0.001$ , 240 vs. 90 min  $P < 0.001$ ; RBC 120 vs. 90 min  $P < 0.001$ , 240 vs. 90 min  $P < 0.001$ ; casts 120 vs. 90 min  $P < 0.001$ , 240 vs. 90 min  $P < 0.001$ ). There were no significant changes for bacteria (bac 120 vs. 90 min  $P = 0.283$ , 240 vs. 90 min  $P = 0.194$ ).

### Urine dipstick analysis

Statistical data for the parameters analyzed are shown in Table 2. Misclassification rates between the different time intervals (120 vs. 90 min, 240 vs. 90 min) were significant for pH (120 vs. 90 min  $P < 0.001$ , 240 vs. 90 min  $P < 0.001$ ), leukocytes (120 vs. 90 min  $P < 0.001$ , 240 vs. 90 min  $P < 0.001$ ), nitrite (120 vs. 90 min  $P < 0.001$ , 240 vs. 90 min  $P < 0.001$ ), protein (120 vs. 90 min  $P < 0.001$ , 240 vs. 90 min  $P < 0.001$ ), ketones (120 vs. 90 min  $P < 0.001$ , 240 vs. 90 min  $P < 0.001$ ), blood (120 vs. 90 min  $P < 0.001$ , 240 vs. 90 min  $P < 0.001$ ), specific gravity (120 vs. 90 min  $P < 0.001$ , 240 vs. 90 min  $P < 0.001$ ) and urobilinogen (120 vs. 90 min  $P = 0.031$ , 240 vs. 90 min  $P = 0.125$ ). Misclassification rates were not significant for glucose and bilirubin (glucose 120 vs. 90 min  $P = 0.250$ , 240 vs. 90 min  $P = 1.000$ ; bilirubin 120 vs. 90 min  $P = 1.000$ , 240 vs. 90 min  $P = 0.125$ ).

**TABLE 1.** Urine particle analysis parameters in three different time points.

Parameter (N = 321)	T0	T1	T2	P (T0 vs. T1)	P (T0 vs. T2)
Conductivity (mS/cm)	14 (10.6 – 18.3)	14.1 (11.0 – 18.5)*	14.8 (11.9 – 19.1)	P < 0.001	P < 0.001
WBC (/μL)	72 (24 – 343)	64 (22 – 317)†	57 (20 – 306)	P < 0.001	P < 0.001
RBC (/μL)	39 (14 – 267)	35 (11 – 211)	27 (9 – 193)	P < 0.001	P < 0.001
Epithelial cells (/μL)	11 (5 – 22)	11 (5 – 21)	11 (4 – 23)	P = 0.610	P = 0.041
Bacteria (/μL)	142 (14 – 1950)	170 (19 – 1658)	181 (19 – 1949)	P = 0.283	P = 0.194
Casts (/μL)	1 (0 – 2)	1 (0 – 2)	1 (0 – 2)	P = 0.009	P < 0.001

Data are presented as median and interquartile range (IQR). The Friedman test was used to test the differences in urine particle analysis for urine particles analysed in the respective analysis time points: T0 - within 90 min, T1 - 120 min after collection and T2 - 240 min after collection. WBC - white blood cell count. RBC - red blood cell count. P < 0.05 was considered statistically significant.

\*N = 309 (12 samples missing).

†N = 320 (1 sample missing).

**TABLE 2.** Misclassification rates for urine dipstick analysis.

Parameter (N = 321)	T0 vs. T1	T0 vs. T2	P (T0 vs. T1)	P (T0 vs. T2)
pH	27 (8.4)	33 (10.3)	P < 0.001	P < 0.001
Leukocytes (/μL)	45 (14.0)	31 (9.7)	P < 0.001	P < 0.001
Nitrite (pos/neg)	12 (3.7)	17 (5.3)	P < 0.001	P < 0.001
Protein (mg/dL)	22 (6.9)	30 (9.3)	P < 0.001	P < 0.001
Glucose (mg/dL)	1 (0.3)	3 (0.9)	P = 0.250	P = 1.000
Ketone (mg/dL)	29 (9.0)	28 (8.7)	P < 0.001	P < 0.001
Urobilinogen (mg/dL)	6 (1.9)	4 (1.2)	P = 0.031	P = 0.125
Bilirubin (mg/dL)	1 (0.3)	4 (1.2)	P = 1.000	P = 0.125
Blood (/μL)	37 (11.5)	64 (19.9)	P < 0.001	P < 0.001
Specific gravity	35 (10.9)	57 (17.8)	P < 0.001	P < 0.001

Shown are misclassification rates (absolute numbers and percent (in brackets)) and P values (McNemars' tests) for urine dipstick analysis parameters pH, leukocytes, nitrite, protein, glucose, ketone, urobilinogen, bilirubin, blood and specific gravity for the respective time points (T0 - within 90 min, T1 - 120 min after collection, T2 - 240 min after collection, T0 vs. T1 - within 90 min in comparison to 120 min after collection, T0 vs. T2 within 90 min in comparison to 240 min after collection).

## Discussion

The present study shows that significant changes occur during automated urine particle analysis of the parameters WBC, RBC, casts and conductivity over the course of the respective time points of measurement. A significant decrease in concentra-

tions of RBC, WBC as well as casts can already be noted between 90 and 120 min. A significant increase in conductivity was found over the course of the respective time points of measurement. In automated urine particle analysis no significant

changes were noted regarding bacteria. In some patients, we detected a decrease in bacteria populations, while in others, we found an increase. For the following parameters of the urine dipstick analysis, significant misclassifications over time were found: specific gravity, pH values, leucocytes, blood, nitrite, ketones, protein and urobilinogen (only at 120 min after collection). No significant changes were found for glucose and bilirubin. In line with results from automated urine particle analysis, both measurement methods showed a decrease in WBC and RBC; significant misclassifications were also found for blood and leucocytes in urine dipstick analysis. For clinical assessment, it has to be noted that the protein pad of the urine dipstick analysis detects mainly urine albumin. The CLSI Guidelines state that optimum analysis is only possible with adequately collected analysis material that has been correctly transported to the laboratory. The guidelines claim that, while urinalysis should be performed as rapidly as possible, analysis can be done up to two hours after sample collection during which time the sample is stored at room temperature (1). Our results show that already at a time shorter than 120 min from urine collection, differences are detectable in the individual measurement results. The question arises whether this is clinically relevant. In urine particle count, the combination of leukocyturia and erythrocyturia is generally used to confirm the diagnosis of urinary tract infection, while in urine dipstick analysis diagnosis is confirmed by positive leukocyte and blood test pads (15). In contrast to earlier studies with UF100™ (Sysmex), where RBCs were not significantly decreased after up to three days storage at room temperature without preservatives, the present study reveals a significant decrease in this parameter over time (16). Thus, a potentially false negative finding can occur when analysis is delayed. The inability of exact WBC classification can cause problems in clinical interpretation. Depending on the tentative diagnosis, exact classification in neutrophil granulocytes (bacterial infections) (17), eosinophil granulocytes (e.g. acute interstitial nephritis) (18) or lymphocytes (e.g. transplant rejection) would be beneficial (19). Urine dipstick analysis reveals ester activity of

granulocytes. Here, the label 'leukocyte' for the respective test pad may be misleading for the clinician, since it only refers to neutrophil granulocytes. Incorrect interpretations of the obtained test findings at a later point in time may not only result in undertreatment but may also lead to increased costs of analysis due to implausible results. In the US, the annual cost of antibiotic treatment of UTI is estimated to be above one billion dollars (20). Based on our results, we recommend that urinalysis is not only performed as rapidly as possible according to CLSI Guidelines, but optimally within 90 min from sample collection. The time point of 90 min was chosen as it represents a feasible time period in the current daily routine of sample collection and transport between the various wards and outpatient departments and the central laboratory at the University Clinics Bonn, Germany. At the University Clinics' Bonn central laboratory, we found that a time period of 90 min between collection and analysis is just about realizable. In Germany, registered doctors' surgeries and many hospitals are now relying on services from laboratories that are located at a considerable distance. Thus, the required time of 90 min between sample collection and analysis is either difficult to achieve or not feasible.

Despite the fact that this is a well-known problem, very little attention is given to preanalytics. Yet, in the event of clinically implausible results, a possible preanalytical bias should definitely be considered. In the very common disease pattern of UTI, consequent adherence to correct preanalytics is problematic for clinicians. Also, there are no standardized reference ranges. Moreover, different cut-off values currently exist. In a study with 438 samples analyzed with UF-100™ (Sysmex, Norderstedt, Germany), cut-off values for erythrocytes (RBC) were 30 RBC /  $\mu\text{L}$  and 41 WBC /  $\mu\text{L}$  (21). In a collective of 2010 samples, the cut-off was 25 cells / mL for evaluation of pyuria and 100,000 bacteria / mL for evaluation of bacteriuria (22). In a study with 252 samples comparing automated analysis (UF-100™) with urine microscopy, cut-offs were 26 WBC /  $\mu\text{L}$ , 26 RBC /  $\mu\text{L}$ , 1.95 squamous epithelial cells /  $\mu\text{L}$  and 0.39 casts /  $\mu\text{L}$  (23). This highlights the problems in urinalysis and the risk of possible false



or delayed diagnosis resulting from the many potential pitfalls. Since UTI is one of the most common bacterial infections, a large number of patients may receive a false diagnosis due to the frequency of this disease (24). Urine dipstick analysis is applied as a simple screening tool and, normally, more elaborate examinations are carried out only in case of pathological findings. Our study shows that the time of analysis after sample collection plays a more important role than previously assumed.

A recent study investigated whether uropathogens are detectable with flow cytometry prior to culture analysis. The aim of that study was to obtain precise, cost effective and rapid reliable results to confirm the very common tentative diagnosis of UTI (25). A weakness of the study was that the time of analysis was within three hours. A study design with an early time of analysis would have been more interesting since we were able to show a noticeable change in many parameters as early as 90 to 120 min from urine collection. An Italian multicenter study carried out a similar investigation. However, UF-1000i™ analysis was performed within four hours after collection (26).

While no significant changes were found for bacteria within the time period of our study, the pathogen spectrum should be considered for clinical interpretation since in some samples we noted an increase and in other samples a decrease. It should also be investigated whether these correlate with the respective pathogen. The most common pathogens of an uncomplicated UTI in women are: *Escherichia coli*, *Enterococcus faecalis*, *Staphylococcus saprophyticus*, *Klebsiella pneumoniae* and *Proteus mirabilis* (27). In the future, microbiologic analysis should be able to determine which bacteria are actually multiplying in the collected urine sample. To include as many pathological findings as possible, specification of criteria of automated urinalysis is becoming more exact, aiming for the lowest possible missed diagnosis rate (28). In addition to the widespread use in the diagnosis of UTI, we also noted significant misclassification rates over different time points of analysis regarding protein concentrations in the protein pad of the urine dipstick where mainly urine albumin is detected. The

possible effects of false negative protein values for patients must be discussed since albuminuria/proteinuria could, for instance, indicate a possible diagnosis of kidney damage (29).

Clinical routine and laboratory diagnosis are crucial and in case of discordances, the analysis methods must be scrutinized and examined for potential sources of error to avoid erroneous therapy. The manufacturer's instructions of the analysis systems that we used list a large number of potential interference factors, which can compromise the correct interpretation of the results. For example, the erythrocyte pad result of the urine dipstick can be false positive due to contamination with menstrual blood or after strenuous physical exercise. Ketones results can be falsified due to fever or fasting and leukocyte results can be distorted by vaginal secretion.

In contrast to Veljkovic *et al.*, who also highlighted the critical issue of the correct time point for urine analysis, our study shows that even earlier time points for analysis should be observed (11).

One limitation of our study was the fact that we did not examine the pathogen spectrum of the bacteria positive results and whether a correlation exists between the specific pathogen and an increase / decrease in number of bacteria. Also, we used Urisys™ 2400 (Roche Diagnostics GmbH, Mannheim, Germany), an older model, which, while no longer produced, is still widely used for diagnosis in laboratories. The lack of universally valid reference ranges is a further problem. Thus, a laboratory must establish its own reference ranges. This calls even more for exact adherence to preanalytics, as confirmed by our results. However, the development of reference ranges can also be problematic since it requires a collective of healthy persons who are not easy to find in the hospital setting. Therefore, reference ranges for a large collective are urgently required for automated urine diagnoses. For the UF-1000i™, there are no German EQA programmes available.

The importance of correct analysis results becomes obvious when considering the broad range of medical problems where urinalysis plays a decisive role. Ultimately, our study shows that the

longer the time period between urine sample collection and analysis, the higher the probability of false interpretation due to instability of some parameters. This applies especially to physiological and pathological cell counts, which are decisive in

further diagnosis. Our study stresses the importance of adherence to early time points in urinalysis (within 90 min from collection).

### Potential conflict of interest

None declared.

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