Stability of common analytes and urine particles stored at room temperature before automated analysis

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Summary

Background. **Central outpatient laboratories may need to process large numbers of urinary samples that arrive 4-6 hours after collection. Furthermore, in certain settings, clinics may have difficulty assuring that the urine arrives at the laboratory in refrigerated containers. The aim of this study is to assess the stability of urine samples stored at room temperature for delayed automated dipstick analysis and automated particle examination.**
Methods. **We considered 300 samples collected from adult outpatients. Urinalysis was done at intervals of 0, 2, 4 and 6 hours (+15 minutes) after collection. For chemical examination 200 samples were tested using an automated analyser and strips supplied by Menarini and 100 samples were tested using an automated analyser and strips supplied by Siemens. For particle analysis 250 samples were tested using a Sysmex UF 100 analyser and 50 using an Iris iQ 200 analyser.**
Results. **Among the analytes on test strips the authors observed a statistically significant diminution in samples positive for proteinuria. For particle analysis we observed a significant decrease in the number of casts, RBC ad WBC were quite stable. A significant increase of samples with high bacteriuria was observed in samples stored at room temperature for up to six hours.**
Conclusions. **Data obtained in this study suggest that urine samples could be stored at room temperature for up to 2 hours without significant variation in the results of the physical-chemical and particle analysis. With a longer storage period we observed a decay in quality of the samples: bacterial overgrowth, lysis in RBC, WBC and casts.**
Key Words. **Urinalysis, Dip-strips readers, automated particle examination, storage, delayed testing.**

Introduction

European Urinalysis Guidelines suggest that for many chemical components examined with test strips no preservatives are needed if analyses were performed within 24 hours and tubes were refrigerated. Particle examination specimens, however, should be refrigerated if not examined within 1 hour, 2 hours for microbiological examination[1]. NCCLS Guidelines for Urinalysis and Collection, Transportation and Preservation of Urine Specimens suggest that after two hours at room temperature the chemical composition of urine changes due to the instability of some of the components (bile pigments), consumption of glucose, pH changes, lysis of casts, erythrocytes and leukocytes in specimens with low specific gravity[2].

It is very difficult to act according to these recommendations in a setting characterised by large centralised laboratories and a great number of outpatient's collecting sites with wide territorial distribution. In this context a central outpatients laboratory might end up processing large numbers of urinary samples that arrive 4-6 hours after their collection. To minimise the

Ricevuto: 31-03-2008 Accettato: 14-05-2008 Pubblicato on-line: 01-08-2008

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Table I. Analytical equipment adopted by each Clinical Pathology Laboratory.

Service	Equipment	Strips	Particles examination
A	Menarini Aution Max	Uriflet 9ub	Sysmex UF-100
B	Menarini Aution Max	Uriflet 9ub	Sysmex UF-100
C	Siemens Clinitek Atlas	LAS-10	Sysmex UF-100
D	Menarini Aution Max	Uriflet 9ub	Sysmex UF-100
E	Menarini Aution Max	Uriflet 9ub	Sysmex UF-100
F	Siemens Clinitek Atlas	LAS-10	Iris iQ 200

Equipment for chemical urine examination and sediment evaluation adopted by the six Clinical Pathology Services participating in the study: Aution Max supplied by A Menarini Diagnostics Firenze Italy, Clinitek Atlas supplied by Siemens Diagnostics Medical Solutions, Tarrytown, NJ USA, UF 100 supplied by Sysmex Europe, Norderstedt Germany, iQ 200 supplied by Iris Diagnostics, Chatsworth CA USA.

impact of these questions regarding the quality of urinalysis, some authors suggested the use of chemical preservatives or the refrigeration of the samples. For example, there is evidence to suggest that urine samples stored at +4°C for up to four hours should be suitable for a correct evaluation of formed elements by using optical microscopy or flow cytometry[2,3] and that for the majority of chemical analytes a correct evaluation is possible using test strip analysis after 24 hours of storage[4]. For longer storage the use of a fixative medium for microscopic evaluation[5] or chemical preservative solution such as borate for microbiological examination[6] has been proposed.

The aim of this study was to evaluate the effect of storage, for up to six hours at room temperature, on urine samples before performing chemical analysis using automated test strip equipment and an evaluation of formed elements using automated analysers.

Materials and Methods

In this study, planned by GSEU (Urinalysis Study Group of the Italian Society of Laboratory Medicine: SIMeL), six Clinical Pathology Services in North Italy were involved. Each service collected fifty urine samples, which were tested immediately after collection (time 0) and after two (t-2), four (t-4h) and six (t-6h) hours of storage at room temperature. For each interval a variation of + 15 minutes was allowed to perform urinalysis.

All the samples were collected from adult outpatients using a middle stream technique following the European Urinalysis Guideline recommendations. Patients were instructed, using written and illustrated guidance, in order to obtain adequate samples. These instruction sheets underline the following aspects: wash outer genital area without using any disinfectants, wipe dry, discharge the first portion and collect the midstream in a clean container (usually a plastic cup or a conical tube supplied by the Laboratories), avoid touching the inside of the container, wipe the external surface of the container dry, secure the lid or transfer the urine to the tube[1]. Urine specimens were collected inside each Laboratory in clean disposable 12 mL conical-tubes adopted for urinalysis and stored at room temperature.

Description of analytical methods and equipments used by each Clinical Pathology Laboratory is reported in Table I. For strip analysis 200 samples were tested using an analyser and strips supplied by A Menarini Diagnostics Firenze Italy, and 100 supplied by Siemens Diagnostics Medical Solutions, Tarrytown, NJ USA; we considered the following parameters: esterase, nitrite, Hb, albumin, specific gravity, glucose, ketone bodies, pH, bile pigments (both bilirubin and urobilinogen); the characteristics of the test strips are reported in Table II. Calibration for dry chemistry analysers was secured following manufacturers' recommendations and by using devoted materials: Menarini Auto Check I and II for Aution Max analyser and for Clinitek Atlas analyser. For dry chemistry analysers' lining up Menarini Auto Check I and II was adopted by using Passing-Bablock analysis and MEDCALC version 9.3.90 software.

Two different commercial analysers were adopted to perform urine particle analysis: 250 samples were tested using the Sysmex UF 100 analyser, supplied by Sysmex Europe, Norderstedt Germany, and 50 samples were tested using the Iris iQ 200, supplied by Iris Diagnostics, Chatsworth CA USA. Sysmex UF 100 is an automated analyser performing sediment urinalysis using flow citometry. Casts, cells and bacteria are measured by electrical impedance for volume, forward light-scatter for size and fluorescent dye for nuclear and cytoplasm characteristics[7]. IL iQ200 is an automated urine microscopy analyser using a digital image acquisition system and software that is able to recognise the various particles present in urine sediment and to classify these particles in categories[8]. Both the instruments give quantitative results. In particle examination, we considered the following parameters: leukocytes (WBC), erythrocytes (RBC), squamous epithelial cells (SEC), casts, (CASTS), bacteria (BACT), non squamous (small) epithelial cells (NSEC). For automated particle analysis, calibration was secured following manufacturers' recommendations and by using devoted materials: Sysmex UF Check for UF-100 analysers and IQ calibra-

Table II. Chemical parameters studied by dip-strip, reaction principles.

Parameter	Reaction principle	Cut-off values
Esterase	Indoxyl esterase activity	25 WBC x 10^6/L
Nitrites	Griess reaction with azo dye	Absent
Haemoglobin	Pseudo peroxidase activity by the haem moiety of haemoglobin	0.00 - 0.02 mmol/L
Albumin	Non specific binding to indicator dye	0.00 - 0.10 g/L
Relative density	Ionic solutes in urine reacts with poly electrolytes bounded on the strip	1009 - 1028
Glucose	Glucose oxidase + peroxidase	0.00 - 0.55 mmol/L
Ketone bodies	Nitroprusside reaction	Absent
PH	Two indicator dyes giving a pH range from 5 to 9	5.5 - 6.5
Bilirubin	Azo reaction with a diazonium salt	Absent
Urobilinogen	Azo reaction with a diazonium salt	Absent

Principle reaction adopted in urinalysis using commercial dip-strip tests.

tion pack and IQ Control Focus Set for iQ200 analyser.

To classify a sample as positive for WBC, RBC and bacteria, we considered the following cut-off values: 25 x106/L for Leukocytes, 15 x106/L for Erythrocytes, 3000/microliter for bacteria; using automated analysers for particle analysis, for dip-stick analysis we adopted cut-off values suggested by the manufacturers[9-12].

For statistical analysis data were evaluated using the software Analyse-it Version 2.03. For descriptive analysis we considered parametric and non parametric statistics, to study the correlation between two variables we considered the Spearman's rank correlation. For means' comparison we considered t-Student test for coupled data (significant with $p<0.05$). For proportions' comparison we considered Pearson's chi-square (significant with $p<0.05$).

Results

For the chemical strip analysis we considered 300 urine samples, 200 evaluated using analysers and strips supplied by Menarini and 100 evaluated using analysers and strips supplied by Siemens. As shown in Table II, for both analysers the chemical reactions used in dip-strip analysis were identical. As reported in Table III no statistically significant variation was observed during storage in concentration of the considered analytes. A significant decrease ($p<0.05$) was observed in the percentage of samples showing albuminuria over 0.05 g/L: 49% at t-0, 39% at t-2h, 34% at t-4h and 32% at t-6h. For nitrites we evaluated the prevalence of positive samples at t-0 (4%), at t-2h (4%), t-4h (8%) and t-6h (10%): this difference was statistically significant ($p<0.05$) for t-4h and t-6h versus t-0h and t-2h.

For particle analysis we considered 300 urine samples: 250 evaluated by using UF-100 cytometer and 50 evaluated by using Iris IQ 200 analyser. We considered only UF-100 data for bacteria enumeration and quantification obtained by both analysers for: WBC, RBC, SEC, NSEC, CASTS, results were reported in Table IV. A statistically significant decrease in the absolute count was observed only for casts ($p<0.01$) and erythrocytes ($p<0.05$), between the values observed at t-0 and t-6h. Although the count of bacteria increased constantly during the four intervals of evaluation, this difference wasn't statistically significant, even between the two extreme points of observation. However, when the percentage of positive samples was considered instead of the absolute value, we found a significant increase in prevalence of positive samples observed at t-0h versus t-6h for bacteria ($p<0.05$) and nitrites ($p<0.05$). These results are reported in Table V.

For some parameters (nitrite, bacteria, esterase, leucocytes, Hb and eritrocytes) we also considered the variation over and under the cut-off values adopted in this study, false positive (FP) and false negative (FN) samples obtained at t-0h and t-6h are reported in Table VI. As shown, there were a significant number ($p<0.05$) of false positive results for nitrites and bacteria and of false negative results for Haemoglobin.

Discussion

Patients' selection. In this study we considered only adult outpatients because only for these patients it was possible an effective control concerning time and procedure of samples collection. Moreover in Italy, for inpatients urine examinations, such as other routine analysis, are performed in hospital based laboratory. Only for outpatients, in North Italy, it is evaluated the feasibility of large non hospital-based laboratory.

Dip-Strip Analysis. In our experience, for none of the considered chemical-physical parameters, was it possible to demonstrate a statistically significant variation between fresh samples (t-0h) and those stored for up to six hours (t-6h) at 22°C; these results concord with literature[3,4,13]. We observed 24 samples (8%) with glicosuria at t-0h; no significant variation was observed in glucose concentration or the percentage of positive urine during storage for up to t-6h. For protein quan-

Table III. Variation of chemical urine components evaluated by strip analysis.

Analyte		t-0	t-2h	t-4h	t-6h
	Median	0	0	0	0
Bilirubin micromoles/L	5° perc	0	0	0	0
	95° perc.	1.03	1.03	1.03	1.03
Ketone bodies	Median	0	0	0	0
	5° perc	0	0	0	0
millimoles/L	95° perc.	0 00.85	00 0.85	000.85	000.85
Glucose millimoles/L	Median	0	0	0	0
	5° perc	0	0	0	0
	95° perc.	0013.9	0013.9	0013.9	0013.9
Haemoglobin	Median	0	0	0	0
	5° perc	0	0	0	0
millimoles/L	95° perc.	18.6	18.6	18.6	18.6
	Median	15	15	15	15
Esterase WBC*10^6/L	5° perc	0	0	0	0
	95° perc.	500	250	250	250
Relative density	Mean	1020	1021	1021	1020
	SD	80	95	88	92
	Median	0.05	0	0	0
Albumin g/L	5° perc	0.05	0	0	0
	95° perc.	0.3	0.3	0.3	0.3
	Median	0.2	0.2	0.2	0.2
Urobilinogen mg/dL	5° perc	0.2	0.2	0.2	0.2
	95° perc.	1	1	1	1
pH	Mean	5.68	5.67	5.69	5.71
	SD	0.41	0.42	0.47	0.45

Overall variation observed in each analyte. For pH and Relative density parametric statistic was adopted and results were reported as Mean and Standard Deviation (SD). For analytes in ordinal scale non parametric statistic was adopted and results were reported as Median, 5° Percentile (5° perc.) and 95° percentile (95° perc.).

Table IV. Variation in the corpuscled urine component evaluated by flow-cytometry.

Analyte	t-0	t-2h	t-4h	t-6h
Leukocytes elements/liter	0.036×10^9 + 0.102×10^9	0.035×10^9 $+0.099 \times 10^9$	0.035×10^9 $+0.098 \times 10^9$	0.034×10^9 $+0.095 \times 10^9$
Erithrocytes * elements/liter	0.027×10^{12} $+0.020 \times 10^{12}$	0.023×10^{12} $+0.020 \times 10^{12}$	0.021×10^{12} $+0.019 \times 10^{12}$	0.019×10^{12} $+0.018 \times 10^{12}$
Sq. Ep. Cells elements/microliter	47.1 +50.1	44.8 +51.3	45.4 +53.0	46.9 +50.9
Small Ep. Cells elements/microliter	1.2 +0.6	1.1 +0.8	1.1 +0.6	1.1 +0.7
Casts** elements/microliter	0.29 +0.43	0.22 +0.42	0.19 +0.35	0.15 +0.31
Bacteria element/microliter	2,642 +2,606	2,837 +2.954	3,145 +3,347	3,547 +3,578

* $p < 0.05$
** $p < 0.01$
Overall variation (mean value + 1 Standard Deviation) observed in each considered parameter ; results are expressed in SI units for RBC and RBC or as mean number of elements / microliter for Squamous Epithelial Cells, Non Squamous Epithelial Cells, Casts, Bacteria.
We observed a statistically significant variation between t-o and t-6h for Erithrocytes (p<0.05) and Casts (p<0.05)

Table V. Prevalence of positive samples.

Analyte	t-0	t-2h	t-4h	t-6h
Nitrites	12	12	24	30
Cut-off +	4.0%	4.0%	8.0%	10.0% *
Bacteria	74	75	76	84
Cut-off 3,000/mL	29.5%	30%	30.5%	33.5% *
Esterase	77	77	75	71
Cut-off 15 U	25.5%	25.5%	25.0%	23.5%
Leukocytes	96	96	96	96
Cut-off $0.025x10^9/L$	32.0%	32.0%	32.0%	32.0%
Haemoglobin	108	108	108	108
Cut-off 0.06/mL	36.0%	36.0%	36.0%	36.0%
Erythrocytes	123	123	120	117
Cut-off $0.015x10^{12}/L$	41.0%	41.0%	40.0%	39.0%

* $p<0.05$
Prevalence of positive samples at t-0, t-2h, t-4h, t-6h for nitrites, bacteria, esterase, leukocytes, haemoglobin and erythrocytes

Table VI. False positive and False negative results observed by comparison of data obtained at t-0h and t-6h.

Particle	FN n°	FN %	FP n°	FP %
Nitrites	0	0	18	6*
Bacteria	0	0	12	4*
Esterase	7	2.3	1	0.3
Leukocytes	1	0.3	1	0.3
Hb	2	0.6	2	0.6
Erythocytes	8	2.6*	2	0.6

* $p<0.05$
Number and prevalence of false negative and false positive results observed by comparison of data obtained at t-0h and t-6h.

tification we observed a slight although not statistically significant reduction in protein concentration between t-0 (Median 0.05 g/L) and t-2h (Median 0 g/L), moreover we observed a statistically significant ($p<0.05$) decrease in the percentage of samples showing proteinuria over 0.05 g/L. This result is in disagreement with data by Froom and co-workers, which report an increment of proteinuria in samples with initial low levels of protein, and the possibility of false positive results[4]. In our case record, we observed a statistically significant increase in the percentage of samples positive for nitrites, from 4% at t=0h to 10% at t=6h ($p<0.05$). In our opinion, this fact was due to a in vitro bacterial growth during storage at room temperature without any preservative[6]. In dip-strip analysis, haemoglobin is detected by using the pseudo peroxidase activity of the haem molecule and both false negative and false positive results are possible[14-16]. In our study we observed a good level of stability in haemoglobin detection during storage at 22 °C, without significant modification for t-6h.

Particle analysis. Microscopic urine sediment analysis has been the gold standard for examining urine cells and particles. However, it is imprecise and has wide interobserver variability, moreover, it is labor-intensive and time-consuming[16-18]. Automation seems the answer to the need to improve both the accuracy and the productivity of urine sediment analysis. For this purpose, fully automated walk-away instruments such as Sysmex UF-100 and Iris iQ200, that perform automated particle urinalysis, were developed recently. It is now well established that this automated equipment for particle urinalysis are able to supply affordable and standardised results[3,7,11,18], but this equipment is quite expensive and available only in centralised laboratories.

In this study we observed that WBC preservation succeeded better than expected during storage: the mean count was $0.036x10^9/L$ at t-0h and $0.034x10^9/L0$ at t-6h, moreover the prevalence of samples with a number of WBC over the cut-off value was 32% at t-0h and t-6h. We experienced some difficulty in RBC conservation. The mean count observed at t-0h

($0.027x10^{12}$/L) was significantly higher than the count observed a t-6h ($0.019x10^{12}$/L). These data underline the fragility of RBC in urine. Lysis of RBC is particularly critical for counts around cut-off values, as a matter of fact we observed a slight but not significant decrease in prevalence of samples positive for RBC until storage (from 41% at t-0h to 39% at t-6h). In our experience, both large squamous epithelial cells and small epithelial cells were well preserved in automated particle analysis performed up until t-6h. Urinary casts showed a significant instability during storage. In literature there are contrasting reports regarding cast stability during short time storage without preservative solution. To explain our findings, we think that in our case record the great majority of observed casts are hyaline casts, that are particularly subject to lysis even in preservative solutions. Moreover repeated automated analysis requiring the aspiration of the samples may be the cause of the lysis of the casts[2,5,18]. As expected, in urine samples stored at room temperature without any preservative solution we observed a bacterial growth; the mean particle count was 2,642/microliter at t-0h and 3,547/microliter at t-6h but this difference did not reach statistical significance because of the very high standard deviation.

Bidirectional changes in particle concentration

During a preservation period, increases and decreases in particle counts may occur, both of which may be clinically significant. These changes may not alter the mean counts for particles at given time points because alterations in the opposite direction may compensate each other. For some parameters: RBC and Hb, WBC and esterase, bacteria and nitrites; we determined the degree of clinically significant bi-directional changes in the classification of the samples - i.e. from positive to negative and simultaneously from negative to positive during the preservation time. These results were analysed by grouping them in two categories at each clinical cut-off value without any grey zone. We observed a dissociation between haemoglobin concentration, that gave us a stable result - 36% of positive samples from t-0h to t-6h - and RBC count, that showed a decrease in percentage of positive samples from 41% at t-0h to 39% at t-6h.

Prevalence of samples with WBC count over the cut-off value was constant: 32% from t-0h to t-6h but we observed a slight decrease in prevalence of samples positive for esterase.

Samples with a bacteria count over the cut-off values showed a significant increase at t-6h but not at t-4h in respect to t-0h. Moreover, as reported in Table VI, for these parameters we evaluated FN and FP results by comparison of data obtained at t-0h versus data obtained at t-6h. For nitrites and bacteria only FP results were observed (6% and 4% respectively). For Hb and erythrocytes we observed both FN and FP results (FN: 0.6% and FP 0.6% for Hb; FN: 2.6% and FP: 0.6% for RBC). For esterase and leukocytes we observed both FN and FP results (FN: 2.3% and FP: 0.3% for esterase; FN: 0.3% and FP: 0.3% for WBC). These findings are explainable by considering that lysis of RBC or WBC could be responsible of false-negative (FN) results, while increases in counts may result from bacterial growth, precipitation of new particulate material, or misclassification caused by in vitro changes in particle size or granularity, leading to false-positive (FP) results.

As conclusive consideration, data obtained from this study suggest that urine samples could be allowed to be stored at room temperature for up to 2 hours without significant variation in the results of the physical-chemical and particle analysis. For a more extended storage we observed a decay in samples' quality: bacterial overgrowth, lysis in RBC, WBC and casts. For samples requiring storage for more than four hours the use of chemical preservatives and refrigeration will be recommended.

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