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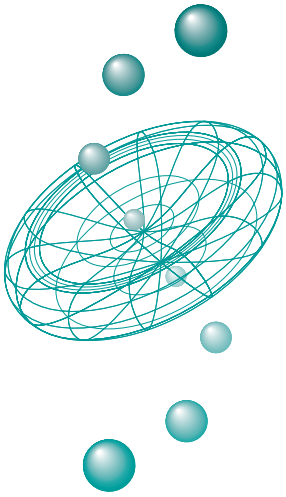

SERIES
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The Effect of Delay in Processing on Urine Particle Analysis

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INTRODUCTION

The essential foundation for correct diagnosis and consequently suitable therapy is the analysis result produced in the hospital laboratory. Depending on the individual analysis to be determined, a variety of different body fluids may be submitted to the laboratory for analysis. These body fluids vary widely in their characteristics, stability and on the individual parameter to be determined. This article deals with urine which is a decidedly hostile environment especially for particle components. Therefore, any delay in carrying out particle analysis after micturition has to be regarded as very important.

THE PROBLEMS

By the time the urine specimens arrive in the laboratory for analysis, it is rarely known how much time has elapsed since micturition. Sometimes, the specimen containers have already been standing on the wards for several hours before transportation to the laboratory. High workload with a low number of staff in the laboratory can prolong this time interval even more.
Analysis of particle components present in urine is not without reason. Not only dissolved substances, such as proteins, are important indicators for serious diseases of the kidney and urinary tract, but the same applies to casts and, of course, cells such as erythrocytes (their morphology is especially relevant to locate the origin of haematuria) and the different types of epithelial cells, *e.g.* renal tubular cells, transitional epithelial cells, etc. and their differentiation from one another. Another common request is for the diagnosis of urinary tract infection (UTI), in which leukocytes, bacteria and (more rarely) yeast cells play a role. In patients suffering from urolithiasis, the presence of certain crystals in the urine may be of diagnostic relevance [1-3].
As a urine specimen ages, the risk of not detecting certain particles increases at all because they are completely destroyed. The same holds true for possible misclassification of cells because they have changed their morphological appearance due to altered environmental conditions. Overlooking, misidentification, over-estimation or under-estimation of a certain particle population, are analytical errors which can lead to a wrong diagnosis. This could have been prevented, had the urine been analysed at an earlier point in time. Thus, according to the current guidelines, documentation of the exact time of micturition forms part of the correct pre-analytical procedure and should be included in the specimen identification detail[1, 4].

WHAT IS THE CORRECT TIMING?

Information found in the literature is variable, nevertheless, on the whole, some consensus is found. Judged as

ideal are time periods of 30 min. [5], 30-45 min. [6], 30-60 min. [7] and 1 h [1], calculated from the time of micturition, within which urinalysis should be performed. In practice, it is often simply not possible to keep within these recommendations; therefore it is useful to know that generally delay maxima of 1-2 h[4, 6, 8, 9] from the time of voiding until analysis seem acceptable.

Depending on the environment prevailing for each specimen and the storage conditions (these will be discussed more in detail below), the time until decay of formed elements begins may generally be from 2-4 h at temperatures of 2-8°C[1, 4, 5, 8]. For erythrocytes, however, decay may not commence for up to 6 h[7]. On the other hand, within 3 h on average already 50% of the WBC initially present are lysed[7] and the WBC count determined after 2 -4 h is considered questionable[1]. Hyaline and granular casts are generally not stable for long[7]. Exceeding 2 h results in certain loss of these elements. When refrigerated the number of bacteria has been reported to remain stable for up to 24 h[1, 7]. The subject of refrigeration will be developed later.

The general view in the scientific literature is that the addition of preservatives to urine specimens be discouraged, since these may interfere with the analysis in many different ways[1, 4, 6-9]. Thus, the time intervals quoted above should not be exceeded, as a prolongation of the storage time for urine specimens cannot be achieved without diminishing the value of the analytical result.

GENERAL DETRIMENTAL INFLUENCES DUE TO DELAY IN PROCESSING

With delay in urine processing, photolytic, oxidative and hydrolytic processes continue, leading to chemical degradation. In addition to decay of cellular components, dissolved analytes such as ketones[7] and light-sensitive substances such as urobilinogen and bilirubin[7, 9] become involved. For this reason storage of urine in the dark is recommended. This may also slow down the considerable darkening of urine colour, which progresses under the influence of light and time[7, 9]. In addition, leaving the urine specimen to stand results in loss of the characteristic odour compounds[9].

Listed below are a few examples of influences on urine particle analysis resulting from delay in processing:

- The pathological excretion of uric acid can only be recognised in a fresh urine sample, since uric acid precipitates spontaneously on leaving the urine to stand[1, 7, 10]. The same applies to calcium phosphate and calcium oxalate.
- Yeast cells develop pseudo-mycelia and this may conceal other cells present in the sample[10].
- Spermatozoa become immobile in old urine and may be confused with parasites[9].
- Trichomonads cease to show any mobility in old urine and cannot be distinguished from leukocytes[9, 10] or may even be confused with renal epithelial cells[7].

A further phenomenon, which may occur on leaving the urine sample to stand in open vials during the course of analysis, *e.g.* after centrifugation or test strip testing, is contamination by airborne particles. In addition to bacteria, fungal spores, pollen and plant cells have all been observed in this context. These particles may enter the laboratory air through an open window[10].

EFFECT OF pH

Physiologically speaking, there is no requirement for stabilisation of cells in urine since normally these are not present. Because of this, urine is not buffered. As a result any strongly acid or alkaline compound in urine will have an unrestricted effect on urinary pH. The physiological pH of urine is usually 5.0 - 6.5 (adults; morning urine), thus being mildly acidic. More extreme values of 4.5 - 7.9 may occur during the course of the day particularly after meals, depending on the type of food consumed; children frequently show alkaline urines[7]. The collection of a urine specimen, with the exception of bladder puncture, usually does not yield sterile material. This means bacteria are practically always present. Bacteria, in the course of their metabolic activity, and depending on the strain, break down glucose and urea. Glucose and urea will thus escape analysis either completely or at least partially. Urea in the presence of certain bacteria is degraded to ammonia by a fermentation process resulting in strongly alkaline urine[1, 6, 9, 10]. This results in a number of disadvantages with regard to particle analysis:

In an acid environment, cells such as leukocytes and erythrocytes, or formed elements such as crystals and casts are more stable[9]; however, an increasing pH has a deleterious effect. Once the urine becomes alkaline, lysis is greatly accelerated[10].

Casts consisting of Tamm-Horsfall protein[7] (a mucoprotein able to form reversible aggregates) dissolve in a short time in urines becoming progressively alkaline as a result of their bacterial content. By the time urinalysis finally takes place, casts may have vanished completely ("bacterial decomposition" of casts) [6, 9].

The presence of crystals in urine is highly dependent on their chemical composition as well as on pH-value and temperature. Amorphous urates, calcium oxalate and uric acid crystals (the last only in acidic urine) are more likely to be found in acidic or neutral urines, whereas amorphous and crystalline phosphates, especially triple phosphates, are precipitated in large numbers in alkaline urine [6, 10]. The presence of such crystals may mask diagnostically relevant cells[4, 10].

Regarding the diagnostically more important crystals composed of an organic matrix, the situation is no better: leucine and tyrosine crystals only appear in acidic urine and dissolve in an ammoniacal environment, which may easily occur in urine after long delay in processing. More sensitive are the rare but diagnostically important cystine crystals, whose appearance in the urine indicate a congenital disorder of the tubular reabsorption of cystine[6] leading to urolithiasis[10]. The hexagonally shaped cystine crystals are only stable up to pH 4.0 - pH 6.0[1, 10] and are only visible in fresh urine. In urine containing bacteria they are rapidly destroyed[6].

Finally, unrestricted multiplication of bacteria present in urine over a longer period of time leads to difficulty in reaching an accurate diagnosis by microscopy[10].

EFFECT OF OSMOTIC PRESSURE

In water- and electrolyte metabolism, osmolality and pH-value are associated with each other, obeying natural laws. A higher pH-value is accompanied by a lower osmolality and a lower specific gravity (SG)[7, 9]. In diluted aqueous solutions specific gravity and osmolality show a certain proportionality to each other; deviations only occurring in urines where higher concentrations of dissolved, organic components (*i.e.* glucose in diabetic specimens) raise the specific gravity but at the same time do not influence the osmolality to the same degree. This is the case when the component in question is not an electrolyte. The reference range for healthy adults (children show lower values) for urinary osmolality lies between 500 - 1,200 mOsm/kg; the specific gravity lies between 1,020 - 1,035[7].

Osmotic effects exert a direct influence on cells. Two liquids in contact with each other separated by a semi-permeable membrane, in this case the urine surrounding the cell and the cytoplasm inside the cell, always strive for osmotic balance. The cell membrane is acting as the semi-permeable membrane in this case.

Urine whose electrolyte concentration is higher than that of the cytoplasm is called hypertonic. As a consequence, water from the cell diffuses into the environment and the cell shrinks. Hypertonic urine is accompanied by an acidic pH-value < 6.0. Erythrocytes in a hypertonic aqueous solution show characteristic alterations in shape through the loss of cytoplasm, taking on a crenated shape.

Urine whose electrolyte concentration is lower than that of the cytoplasm is called hypotonic. Hypotonic urine is especially threatening correct cell enumeration, because, as a consequence, water from the environment enters the cells, which eventually swell and finally lyse. Hypotonic urine is accompanied by an alkaline pH-value > 7.5 - 8.0 and reduces the stability of particles in the urine.

Urine having an osmolality < 300 mOsm/kg, a SG < 1.010 and a pH-value > 7.0[4, 8] is regarded as a hostile environment for cells. Here, lysis of erythrocytes and leukocytes takes place rapidly. Other authors mention values of < 360 mOsm/kg and a SG < 1.009, at which lysis of erythrocytes is strongly accelerated[10].

Erythrocytes in urine are of special importance since their shape may give a hint to the source of haematuria (renal or post-renal), thus indicating also whether the patient has to be treated further by a nephrologist or a urologist. A frequent examination is therefore determination of the erythrocyte morphology by phase contrast microscopy[1]. Changes of the RBC towards a dysmorphic appearance may result from disorders of the glomerulus, but also from osmotic effects[1, 10, 11]. Therefore, even more important are situations when RBCs shrink to crenated shapes (echinocytes, schistocytes) in hypertonic urine or swell to become stomatocytes or ghost cells in hypotonic urine[6, 11]. When this happens, no reliable statement can be made regarding the origin of these RBC[1]. Swollen RBC appear more transparent on microscopy and are less easily seen due to their low refractive index[9]. This may lead to undercounting of these cells[1].

The morphology of leukocytes is also influenced by osmolality. Apart from obvious lysis in hypotonic urine there are some more subtle influences. Neutrophil granulocytes frequently degenerate in diluted urine, resulting in loss of morphological features and change in shape of the cell. In concentrated urine, the cytoplasmic organelles clump merging with the nucleus and there is a danger of misclassifying these cells with small, tubular cells on microscopy[10].

In summary, cell lysis due to osmotic effects must also be regarded as a phenomenon caused by delay in processing due to the shift of pH to alkaline which occurs particularly in bacteria-containing urine.

EFFECT OF TEMPERATURE

The multiplication of bacteria originally present in the urine takes place more slowly on refrigeration. The deleterious effects of osmosis are also slowed at temperatures of 2 - 8 °C.

Casts are unstable at temperatures of 20 - 25°C. Thus, they may escape analysis if the urine specimen is stored at room temperature, even when pH and osmolality show no significant change[8].

Therefore it is advisable to store a urine specimen preferably immediately under refrigeration if speedy transportation and particle analysis is not possible.

Refrigeration helps to prolong the time period over which adequate examination may be undertaken but there are disadvantages such as the formation of precipitates (crystals and amorphous salts from electrolytes and possibly drug metabolites) that may mask diagnostically important particles[4, 10]. Because of this some authors[6, 7, 10] advise against refrigeration.

For these reasons, refrigeration should only be employed when analysis of the specimen is not possible within 2 h of micturition. There is no real substitute for speedy examination of fresh urine. Every effort should be made, both by clinical staff and by laboratory staff to minimize the delay in processing urine specimens.

IS THE EFFORT WORTHWHILE?

There is a general opinion that accurate urine particle analysis possesses severe limitations. Often, results are regarded as merely qualitative or at best semi-quantitative. This will be true so long as pre-analytical variables are ignored.

The determination by conventional manual methods of particle counts in the low concentrations found in urine is subject to high statistical uncertainty[1, 12, 13]. Particle counting under these circumstances follows a Poisson distribution, which results, for example, in a coefficient of variation (CV) of 60% at a particle concentration of 3 / µL and an analysed volume of 1 µL. If 5 µL of the sample is analysed, the CV would still reach a value of 26%. Only when the analysed volume is 10 µL, will the CV drop to 18%, thus permitting the determination of a reliable mean value and count result[1]. A particle concentration of 3/ µL is a not uncommon finding and represents the decision

value for a number of diagnostically relevant formed elements.

In the case of chamber counting, the low volume of urine used is the weak point, as very often, depending on the type of chamber, from 1 - 3.2 µL urine at the most are examined. As a consequence, particle concentrations < 10 - 30 / µL are statistically unreliable[1]. In the case of the urinary sediment, limitations are based on the heavy and variable cell losses due to centrifugation and again on the low volume used for microscopy. In routine analysis often only 2 high power fields (HPF) are looked at, although for a statistically reliable result at least 10 HPF are necessary[1, 5]. The urine volume of one HPF amounts to merely 0.173 mL, in spite of its 20-fold concentrating by means of centrifugation[1]. Centrifugation leads to significant cell loss due to non-sedimentation, especially RBC and WBC (up to 50 %), and casts, which fragment due to the mechanical strain during centrifugation[1, 12, 13].

Many of the difficulties described above may be eliminated by Urine Flow Cytometry (UFC), first introduced by Sysmex in 1996. The fully automated instruments of the UF-series count and analyse particles from native urine, so that the sources of error inherent in centrifugation are eliminated. The counting volume is 9 µL of urine, so that with this method, statistically representative results can be determined. Also with objective particle classification the subjective influence of the laboratory technician is removed. The analytical performance spectrum of the UF-series is amplified by measurement of erythrocyte size distribution thus offering information on the site of origin of haematuria. The high analytical quality of these systems has been confirmed in many evaluations[2].

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