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NEW SCREENING DIAGNOSTIC TECHNIQUES IN URINALYSIS

J. Delanghe

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ABSTRACT

New technological evolutions have enabled new diagnostic approaches in urinalysis. Urinary flow cytometry and automated microscopic pattern recognition are two new techniques that are characterised by a much better imprecision and a higher throughput as compared to conventional microscopy of the urine sediment. Although these new techniques are well suited for the routine clinical laboratory for screening and diagnostic purposes, trained technicians are still required to verify, and if necessary, to correct the results by visual microscopy. On the other hand, automated urinary test strip analysis offers analytical, clinical, and labour cost-saving advantages. Furthermore, determination of specific urinary proteins offer interesting alternatives for diagnosis. In diabetics, the clinical significance of non-immunoreactive microalbumin needs to be established. Furthermore, the determination of specific urinary proteins alpha 1 microglobulin (as a tubular marker protein), alpha 2 macroglobulin (as a haematuria location marker) and light chains (myeloma monitoring) offer interesting diagnostic perspectives. As the information content obtained by urinalysis is complex, expert systems that make use of the various chemical and morphological parameters can offer an interesting help in the interpretation.

Department of Clinical Chemistry
University Hospital Ghent

Address for Correspondence:
Joris Delanghe
Deaprtment of Clinical Chemistry
University Hospital Ghent – 2P8
De Pintelaan 185 (2P8)
9000 Ghent
Belgium
E-mail: Joris.delanghe@ugent.be

INTRODUCTION

The examination of the urine remains one of the most commonly performed tests in the clinical laboratory. A critical evaluation of sediment analysis techniques has revealed a number of significant flaws, particularly in relation to centrifugation (1), to the type of optics used, and to the failure to stain prior to microscopy. Bright field microscopy allows only poor visualisation of hyaline casts and red blood cells (RBC) with low haemoglobin content; leukocytes may be difficult to differentiate from tubular cells, and bacteria may not be seen or may be confused with amorphous debris. Phase contrast microscopy is a superior method (2). Polarized light microscopy is recommended for the identification of lipids and crystals and immunofluorescence microscopy can be used to identify the protein composition of casts (2).

Chamber counting by phase contrast microscopy of supravitally-stained uncentrifuged urine is considered the best candidate for reference (3-5). The conventional sediment is hampered by the variable but significant particle type-specific loss during centrifugation (1, 3). The large number of manipulative processes in the sedimentation method add to the imprecision. Hannemann-Pohl (6) reviewed the critical steps in sediment microscopy and demonstrated that these result in a coefficient of variation (CV) exceeding 100%.

In view of the poor analytical and economical performance in traditional urinalysis, automation and the search for alternative diagnostic approaches have become a necessity, in particular for screening purposes. Routine urine particle analysis stays historically behind haematology in the development of automation of emergency laboratory services, because urine is a more difficult matrix than blood, particles vary 100-fold in their size (from about 1 to 100 µm in diameter), and because urine particle concentrations are generally low. Recent technological progress in the fields of particle analysis and immunochemistry has enabled to change routine urinalysis in a profound way. In this paper, the various new diagnostic approaches in urinalysis are reviewed.

AUTOMATED URINALYSIS

The first attempt to automate urine microscopy occurred 25 years ago with the development of the Yellow IRIS (7). A dye is added and the stained urine passes through an optical pathway; the particles are captured on a videocamera. The particle images are sorted according to their lengthwise dimensions and presented for validation. Precision and sensitivity are better than with visual microscopy. However the throughput and the level of differentiation are highly variable depending on the particle concentration and observer interpretation (8).

The UA-1000 and UA-2000 (Sysmex) were introduced in 1990 and 1993, respectively (9). These devices edited and analyzed particle images taken by a colour CCD (charge coupled device) camera. They suffered from low throughput and required a trained analyst to assess the images generated.

The Sysmex UF -50 and UF-100 have been developed based on a different concept (10-12). These analysers are high throughput (up to 100 results/hour) flow cytometers designed to automate the recognition and counting of particles in urine. The instrument combines flow cytometry with impedance analysis of urine particles after staining with two fluorescent dyes carbocyanine stains the cell membrane and phenanthridine stains nucleic acids. The particles are hydrodynamically focused; a laser beam encounters these particles and particle-specific forward light scatter and fluorescence signals are emitted. Based on the characteristics of forward scatter, forward scatter pulse width, fluorescence, fluorescence pulse width and impedance, the particles are classified. In the more recent UF1000i instrument the argon laser has been replaced by a semiconductor laser.

Urinary flow cytometers can identify RBC, WBC, squamous epithelial cells, small round cells (this category includes renal tubular cells and transitional epithelial cells), hyaline casts, pathological (inclusional) casts, bacteria, yeast-like cells, spermatozoa and crystals. Results are displayed as scattergrams and histograms and numerical values are reported.

The Iris iQ200 Urine Microscopy Analyzer is a second-generation urine analyzer (Iris Diagnostics). The iQ200 captures images from planar flow of urine particles (13-15) with a CCD camera. The software allows to display the images of particles for visual confirmation. Its neural network algorithm uses particle size, shape, contrast and texture as features to enhance its classification of particles. The automated urinalysis system can be physically and electronically connected to a test strip reader. Specimens are hydrodynamically focused between two layers of suspending fluid (lamina). Lamination is the planar equivalent of axial hydrodynamic focusing, as used in blood cell counters and in flow cytometers. The particles can be seen through the objective lens of a microscope coupled to a video camera. The camera captures 500 frames per sample. Size, shape, contrast and texture features are used by the neural network technology. After autoclassification, an operator may visually confirm the identification or reclassify particles based on their morphology. The operator reviews the images of all abnormal results, and edits them if necessary. The categories reported by the instrument include RBC, white blood cells (WBC), white blood cell clumps, squamous epithelial cells, non-squamous epithelial cells, hyaline casts, non-hyaline casts, bacteria, crystals, yeast, sperm and mucus. Images that cannot be recognised with high confidence are reported as artefacts (unclassified particles). The iQ200 shows good linearity and precision and no carry-over is present. The system is reliable in counting RBC, WBC, and squamous epithelial cells (13-15). It also identifies a fraction of bacteria and renal elements (casts, non-squamous epithelial cells) with the assistance of a trained technologist. Detection of casts fails with the software alone but improves in the reclassification by the user. Although squamous epithelial cells are fairly sharp, small particles may appear out-of-focus and operators cannot focus them better as it is possible in a visual microscope. Technical improvement is needed to improve the specificity of yeast and crystal detection. The lower limit of quantification is about 20 – 30 particles/µl.

Evaluations have established acceptable linearity over useful working ranges, with an imprecision that is significantly less than microscopy. Urine particle flow cytometers (UFC) and automated microscopy instruments offer significant labour saving (10,13) and can be used for screening purposes. Sample carry-over was generally <1% because rinsing steps are automatically performed. The detection limit for most particles is clearly below the upper healthy reference limit.

Additionally, UFC systems provide a flag for RBC size distribution. The location of haematuria can be suggested by urinary RBC morphology (16, 17). The detection of abnormal shapes, such as acanthocytes and other dysmorphic forms, as markers of renal haematuria has become a tradition of erythrocyte analysis by the microscope. Some investigators have studied the use of urinary flow cytometry for assessing the location of haematuria (18). Others have looked at the possibilities for using UFC in the fast diagnosis of urinary tract infections (3, 10, 11) and applications of monitoring and exclusion of renal disease (10).

Uncentrifuged urine counts compared well with UFC counts for RBC and WBC (3, 10, 11). UFC Identification of urinary crystal type requires visual microscopy. In most cases, only pathological crystals such as cystine or 2,8-dihydroxyadenine are of clinical relevance. UFC appears to miss casts with a frequency of about 14% (10). Renal tubular and transitional cells were correctly indicated by UFC in only two thirds of the samples.

The conductivity measurement in urinary flow cytometers is related to the concentration of electrolytes in the urine. There is a good agreement between conductivity, urine density and osmolarity (r=0.8 or higher) (10, 18). Conductivity correlates better with osmolality than the creatinine concentration and therefore might serve as a better indicator of hydration status (19).

Expert systems exist combining both test modalities based on user definable decision rules. The implementation of such a strategy significantly reduces microscopy review and saves time and expense without diminishing clinical utility.

URINE TEST STRIPS

The ready availability and apparent ease of use of dry chemistry techniques has resulted in these being used as screening or sieving procedures. Mechanization of test strip reading by reflectometry and standardization of the reaction time were factors in improving reliability.

The intensity of the reaction colour of the test pad is detected by measuring the percentage of light reflected from the surface of the test pad. The higher the analyte, the higher the colour intensity and, thus, the lower the reflectance. The reflectance value, expressed as a percentage within a range from 100% (white) to 0% (black), is therefore inversely related to the concentration of the analyte in the sample. Specific gravity (refractometry based) and clarity are measured in a flow cell, and colour is rated with a specific algorithm against the blank pad on the strip. Data are expressed in an ordinal scale (as "normal", "negative", "positive", or as nominal concentrations) on the reports. However, it is possible to use the remission data which allows a more sensitive and more quantitative measurement (20). The detection limit of the test strip protein assay was 25 mg/l which allows identification of microalbuminuria in contrast to the classic reading of the strips, which can detect only albumin concentrations 150–200 mg/l or higher (20), hence coming closer to the "urine test strip of the future" (21). Because urinalysis test strips are often used for checking urinary flow cytometry data, there is a need for a more quantitative evaluation of urinalysis test strips.

Test strip analysis plays an important role in urinalysis as such, and the value of test strip urinalysis as a screening method has been thoroughly demonstrated (20). If the test strip results are negative, the urine is deemed normal. Another approach is to combine test strips with UFC for primary screening either always by both methods or by using test strips for analytes unrelated to particles analyzed by UFC (10, 12).

SPECIFIC URINARY PROTEINS

Alternatively, the determination of specific urinary proteins may be very helpful in the diagnosis of nephrological and urological conditions (22). Gel electrophoresis of urinary proteins often lacks sensitivity and reproducibility. The availability of sensitive immunoassays which can be adapted on routine clinical chemistry analysers have made the immunochemical determination of specific urinary proteins an attractive diagnostic alternative.

MICROALBUMINURIA

Microalbuminuria is defined as the excretion of 30–300 mg of albumin/24 h (20–200 µg/min, or 30–300 µg/mg

of creatinine). Microalbuminuria is an established early marker for diabetic nephropathy (22). Urinary excretion of albumin in mild diabetes is complex. A hitherto unrecognized form of microalbumin is not detected by customary immunoassays (23). Filtered albumin is partly degraded to fragments by the proximal tubules, where the albumin is taken up into endosomes. These endosomes merge with lysosomes, where lysosomal enzymes cleave the albumin into fragments. However, the albumin fragments are not detectable by immunoassay. Laboratories now report only the excreted amount of whole, undamaged albumin. In healthy subjects, the results of various urinary microalbumin methods (e.g. immunoassays, electrophoresis, HPLC) agree well (24). In patients with minimal diabetic damage, HPLC values were higher than those obtained by RIA (23). Approximately one half of the findings would have been reported as normal, but some renal damage was apparently present; 33 % of diabetics were found to be normal by RIA but to have increased microalbuminuria as measured by HPLC (24). The term "immunochemically nonreactive albumin" (the difference between HPLC and RIA results) has been coined. The processing by renal lysosomal enzymes results in "nicking", but the albumin molecule is still held together by its disulfide cross-links. The heavily nicked albumin is not immunoreactive and is therefore missed by immunochemical methods. The development of new practical methods to measure the total intact urinary albumin (whether undamaged or nicked) as the first indication of renal damage in diabetes is challenging. The benefit may be earlier detection of incipient damage since the detection of immunochemically nonreactive albumin precedes increases of conventional microalbumin values by a mean of 3.9 years in type I diabetes and 2.4 years in type II diabetes (25). Further study is needed to establish the role of immunochemically nonreactive albumin in clinical practice.

On the other hand, a poorly understood dissociation between total protein and urinary (micro)albumin results may also occasionaly point toward the diagnosis of factitious proteinuria (Munchausen syndrom) (22).

Despite its gaining popularity, microalbumin remains a rather expensive parameter for screening purposes. Potential alternatives could be the application of quantitative reading of common test strips (20) or even simpler sensitive staining procedures (26). Simple nigrosin-dye binding tests allow a cut-off of 50 mg protein/l (26), which offers an excellent possibility for first line screening for microalbuminuria at lowest cost.

The much less frequently used parameter microtransferrinuria has been reported to be a more sensitive alternative for microalbuminuria in diabetics. The senstivity might be related to the fact that the charge of the transferrin is not as much altered by glycation (22).

SELECTIVITY OF GLOMERULAR PROTEINURIA

The proteinuria selectivity index may be used to describe changes of the glomerular permeability for macromolecules in glomerular diseases. Large size plasma proteins (alpha 2 macroglobulin, IgM, IgG) require large pores to pass of glomerular basement membrane. Comparison of the clearance of the high-molecular-weight proteins to that of albumin may be useful in characterization and diagnosis of different glomerular diseases (23).

TUBULAR PROTEINS

Tubular proteinuria results when glomerular function is normal, but the proximal tubules have diminished capacity to reabsorb and catabolize proteins, causing an increased urinary excretion of the low-molecular mass proteins that normally pass through the glomerulus, such as retinol-binding protein, alpha 1 microglobulin, beta 2-microglobulin and clara cell protein (27). Table 1 summarizes the physical and physiological properties of some specific urinary proteins commonly used for assessing kidney function.

The evaluation of renal tubular function is an important issue. Detection of urinary microproteins can help to detect renal abnormalities at an early stage and differentiate the various forms of renal and urological pathology. For many years, beta 2-microglobulin has been a golden standard urinary marker protein. Urinary microproteins are becoming increasingly important in clinical diagnosis. They can contribute in the non-invasive early detection of renal abnormalities and the differentiation of various nephrological and urological pathologies. The 27 kDa glycoprotein alpha 1-microglobulin (A1M) is a promising marker for evaluation of tubular function. Because A1M is not an acute phase protein, is (in contrast to beta 2 microglobulin) stable in a broad range of physiological conditions (28) and sensitive immunoassays have been developed, its measurement can be used for clinical purposes. Unfortunately, international standardisation is still lacking as is the case for beta 2-microglobulin (27).

Table 1. Physical and physiological properties of proteins commonly used for assessing kidney function

Marker protein	Molecular mass (kDa)	Remarks
Albumin	68	Early detection of diabetic nephropathy Immunoreactive albumin
Beta 2 microglobulin	11.8	Tubular marker Not stable at acid pH
Alpha 1 micro globulin	27	Excellent tubular marker Marked stability over a wide pH range No standardisation
Retinol binding protein	21	No sensitive assays available
Immunoglobulin G	150	Selectivity index parameter
Alpha 2 macroglobulin	725	localisation of site of hematuria (post-renal proteinuria)
Kappa , lambda chains	25	Myeloma detection
Free light chains	25	Myeloma detection

Urinary A1M provides a non-invasive, inexpensive alternative for the diagnosis and monitoring of urinary tract disorders (early detection of tubular disorders such as heavy metal intoxications, nephrotoxic drugs, diabetic nephropathy, (29-31), urinary outflow disorders and pyelonephritis) (32). Following chronic ureteral obstruction, an increase in urinary A1M excretion was observed, which is related to the grade of vesicoureteral reflux (32, 34). Early diagnosis of acute pyelonephritis is a challenge. The urinary A1M/creatinine ratio was highly sensitive and specific (34). In contrast to cystitis, elevated urinary A1M excretion is seen in acute pylonephritis (33, 34). Unexpected positive results are found in acute prostatitis (35).

ALPHA 2 MACROGLOBULIN

Alpha 2 macroglobulin is a 725 kDa protein which can be used as a urinary marker for assessing post-glomerular proteinuria. In postrenal bleeding, with albumin concentrations above 100 mg/l, the relative excretion rates of specific proteins are proportional to their plasma concentrations. In glomerular haematurias, however, the ratios to albumin are much lower. The optimal discriminating ratio is 2.0×10^{-2} for alpha 2-macroglobulin/albumin and 2×10^{-1} for IgG/albumin. Tubulointerstitial involvement in haematuria is characterized by elevated alpha 1-microglobulin excretion rates, with alpha 2-macroglobulin/albumin ratios below 2.0×10^{-2} and IgG/albumin ratios above 2×10^{-1}. These basic findings allow the exclusion and differentiation of clinically relevant proteinurias and haematurias (22, 35).

BENCE-JONES PROTEINURIA

Quantitative measurements of urinary paraprotein concentrations play a major role in the monitoring of patients with multiple myeloma. Next to the more traditional measurement of light chain concentrations by the size of the urinary light chain M-spike on electrophoresis or by the measurement of the total (free and bound) light chain concentrations, it is now also possible to measure free light chains (FLCs). The test uses antibodies that specifically recognize an epitope of the common region of kappa and lambda light chains that is “hidden” when the light chains are attached to the immunoglobulin heavy chain (36). The myeloma management guidelines (37) recommend serial monitoring of the FLCs in serum, but periodic 24-h urine collection is still required for Bence Jones proteinuria and total urinary protein quantification. Monitoring of renal involvement and Bence Jones proteinuria in patients with FLC myeloma can be improved by measuring both total urinary protein and FLC in urine (38).

EXPERT SYSTEMS

Expert systems have been developed for checking urinalysis results. Langlois et al (12) have developed an expert system based on a large database of microscopy, test strip and flow cytometry results. The system analyses the UFC and test strip data and identifies results associated with lower reliability and indicates pre-analytical problems. Decision rules are made user-definable. The samples, which need to be reviewed, are given a remark indicating a potential error. The implementation of such a strategy significantly reduced the visual review rate and saved time but without diminishing clinical utility. Microscopy review contributes significantly to the correct diagnosis. The expert systems further need modifications for different individual patient groups (patients with indwelling catheters, patients with renal transplants, diabetic, and paediatric). The application of expert systems has the main advantage that the error rate is greatly reduced and the review rate is kept to a minimum (<15%) (10).

Similarly, various computer-based expert systems have been developed based on a data set including specific proteins, in combination with a test strip screening, the findings of haematuria, leukocyturia and proteinuria, as a decision-supporting tool. In clinical studies, this reached 98% concordance with clinical diagnoses and was superior to the diagnostic interpretations of experts. (39-42).

Assaying urinary A1M provides a non-invasive inexpensive diagnostic alternative for the diagnosis and monitoring of urinary tract disorders (early detection of tubular disorders such as heavy metal intoxications, diabetic nephropathy, urinary outflow disorders, and pyelonephritis). Computer-aided interpretation of parameter sets including A1M as marker for tubular damage may help to improve the quality of the test interpretation.

FUTURE DEVELOPMENTS IN URINALYSIS

UFC and automated microscopy supported by neural networks are interesting and informative technologies which have produced a revolution in urine particle analysis (10,13). The analytical precision is superior to that of visual methods due to the fully automated process. A significant reduction in turn-around time in the laboratory has been demonstrated. Automated particle counting carries a promise of improved detection and quantitative monitoring of diseases of the urinary tract, because visual microscopy is prone to human and technical errors (10). Further progress is expected by expanding the data management possibilities and by consolidating results from various technologies such as test strip, quantitative clinical chemistry, microscopy, immunochemistry and clinical microbiology. This will result in a significant step toward total laboratory automation. Further clinical studies, however, are needed to fully establish fully the diagnostic value of these new parameters.

PRE-ANALYTICAL CARE

Despite the technological progress, the need for standardization of pre-analytical conditions still remains essential for the generation of clinically useful and reliable results. Pre-analytic mistakes include improper specimen collection, storage and transportation, as well as inconsistent and improper handling and manipulation of the specimen prior to analysis (4). In particular the collection phase is important. The sample is preferably obtained using mid-stream catch. Storage at room temperature will promote growth of microorganisms. In order to preserve brittle structures (e.g. casts, Trichomonas), transport time has to be kept short. A cool storage and transport will better preserve the chemical and cellular contents of the sample better. Preservative agents (e.g. boric acid) can be used to increase sample stability (43). Stabilized samples can reliably be analysed for leukocytes and bacteria even a few days after sampling (43). For the other figurated elements, the results are less satisfactory (43). However, cooling may promote crystal formation and adding urinary preservative agents significantly alters the pH value.

CONCLUSIONS

In recent years, the field of urinalysis has undergone a thorough revolution which has affected both cellular and chemical analysis of urine specimens. Imprecison has dropped dramatically which allows a more accurate diagnosis. The technological progress has not been able to completely replace dedicated microscopy, which still has a significance of special tasks and the detection of rare findings. Combining the various techniques results in a higher diagnostic efficiency (44). In the future, the increasing accuracy of the analytical methods will put a larger emphasis on the pre-analytical phase.

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