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APPLICATIONS OF FLOW CYTOMETRY, REFLECTANCE TEST STRIP READING AND SPECIFIC PROTEINS IN MODERN URINALYSIS

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Comments on the cover illustration

The illustrations depict several important milestones in urinalysis.

The painting (from Jan Jozef Horemans, “Graviditas ominosa uroscopia”) describes the Medieval art of ‘piss-pot science’ showing the uroscopist examining urine in a matula.

The bottles represent several possible states and colours of urine from which the uroscopist could deduce the subject’s disease.

The technical drawing illustrates the construction of a modern flow cytometer, while the graph represents a calibration curve of modern urine test strips.

The most exciting phrase to hear in science,
the one that heralds new discoveries,
is not "Eureka!" but
"That's funny..."
ISAAC ASIMOV

Only two things are infinite:
the universe and human stupidity;
and I'm not sure about the former
ALBERT EINSTEIN

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LIST OF ABBREVIATIONS

3HB	3-HydroxyButyrate
A1M	Alpha 1-Microglobulin
A2M	Alpha 2-Macroglobulin
AcAc	AcetoAcetate
CAPD	Continuous Ambulatory Peritoneal Dialysis
CD	Cluster of Differentiation
DKA	Diabetic KetoAcidosis
KDIGO	Kidney Disease: Improving Global Outcomes
LED	Light Emitting Diode
LIS	Laboratory Information System
PCR	Polymerase Chain Reaction
PD	Peritoneal Dialysis
RBC	Red Blood Cell
UPES	Urinary Protein Expert System
UTI	Urinary Tract Infection
VUR	Vesico-Ureteral Reflux
WBC	White Blood Cell

CHAPTER 1

Introduction

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From uroscopy to urinalysis

CHAPTER 1: Introduction - From uroscopy to urinalysis

History of urinalysis

References (1) and (2) deal with this paragraph.

Macroscopic examination of urine for diagnostic, therapeutic and prognostic purposes is as old as medicine itself. Egyptians already could diagnose a diabetic's urine by observing the attraction of flies by urine spilled on the ground. Until the end of the 17th century, urine samples were examined in their natural condition.

Kidney and urinary tract diseases have always fascinated mankind. Early attention to this specific field of medicine was paid by Hippocrates. By his ideas, the interior was ruled by a constant flow of all kinds of fluids: blood, mucus, urine, bile, feces, etc. Thus, the body's secretions were of crucial importance. They were instruments for the perception of the interior while great stress was laid on argument by analogy.

All these ideas were theoretically framed by the doctrine of the four humours which dominated medical thinking for over 2000 years preserving the humoral pathology idea far into the 19th century.

As a tool and diagnostic aid for physicians, uroscopy originated in antique roots, reaching its heights during the Middle Ages. Special attention was focused on the meaning that was attributed to the colour of the urine. Hence, already early on coloured note-boards or urinary glass panes with up to 20 colour grades arranged in columns or circles as mnemonic aids could be found. The colour of the urine indicated the state of the humours in the veins: "Like blood, like urine".

Not only colour was important in uroscopy, early (macroscopic) sediment analysis took into account the consistency and the position of the sediment in interpreting diseases.

Finally, uroscopy became the province of quacks, charlatans and piss-prophets. It revived in the 18th century and regained credibility in the 19th and the first half of the 20th century in parallel with advances in biochemistry. The impressive state of knowledge developed over centuries lost its validity overnight when a modern scientific approach began around 1850 with the quantitative chemical analysis of urine, the introduction of microscopy and later on test strip analysis and flow cytometry.

More than hundred years after introduction of qualitative chemical tests in combination with microscopic analysis of urine, urinalysis is challenged by new medical needs as well as technical developments and advances in scientific knowledge.

Test strip analysis

The revolutionary transition of uroscopy to a more scientific medical discipline took place in the 19th century and is linked to names such as Berzelius, Heller, Scherer and Liebig. They mark the birth of clinical chemistry with classical urine analyses for substances such as protein, sugar, bile acids and hemoglobin (3).

These were originally wet chemistry tests that were not always easy to perform since a substantial amount of time and equipment was needed. Hence, the search for some form of convenient chemistry testing was started early leading first to the development of tablets and later to specific strips. The principle of test strips can be dated back to the invention of litmus paper by Boyle (approximately 1670) or even to the Roman Plinius who described a test for iron by means of papyrus strips soaked in gallae which was later used for human urine. The advantages of the technique of test strips are obvious: it is practicable for general practitioners and even self-testing can be an option.

The modern era of the urine test strip as we know it today started around 1956 (4;5) followed by the instrument reading of test strips in the 1970's. Nowadays, also very sensitive test strips exist or immunological techniques have been introduced for the determination of specific proteins (e.g. albumin, alpha 1-microglobulin (A1M)) (6;7).

From urine microscopy to flow cytometry

The realisation that examination of urine by microscopy could provide vital information occurred only from the 16th century onwards evolving finally into a routine practice, due to improvement of the optics system, in the 19th century. Rayer and Vigla then emphasized the concept that the results of microscopic investigation should always be associated with chemical analysis. This was the period of major breakthroughs concerning the relation between urinary particles and disease (8).

However, urinary microscopy has numerous drawbacks related to centrifugation, optics and staining possibly leading to poor visualisation of some urinary formed elements. Other techniques can overcome these problems e.g. phase contrast microscopy, supravital staining and polarized light microscopy, but in general microscopy has some major drawbacks being an imprecise and laborious technique with a high interobserver variability. These problems have led to the widely used "sieve concept", which states that if the results of chemical screening with dipsticks are negative, then microscopic

examination is unnecessary. Such simplification, however, may lead to substantial losses in diagnostic yield.

Therefore, several attempts have been made to develop automated and more standardized methods to improve both the accuracy and the productivity of urine sediment analysis. The first device occurred in the eighties with the Yellow IRIS (9;10) that captures pictures of native urine on a video camera. Offering a better precision and sensitivity than visual microscopy, throughput was affected by particle concentration and user classification, whereas sensitivity of detection and original particle differentiation depended on the used instrumental algorithm. Moreover, it required continuous monitoring of images by the operator and did not have walkaway capability, necessitating the continuous presence of a system-operator. The advantage of a walkaway analyzer is that it handles the samples totally independent once it is started so no more actions are expected from the user, which is of importance in the ever increasing lab automation. The UA-1000 and UA-2000 (Sysmex Corporation, Kobe, Japan), based on the same principle, were introduced in the early nineties. They still suffered from low throughput and required a trained analyst to assess the images. To face the demands for a fully automated high throughput analyzer, the Sysmex UF-100 was developed based on a different concept. This analyzer is a flow cytometer designed to automate the recognition and counting of particles in urine. It classifies particles based on fluorescent intensity (cells are stained with 2 fluorescent dyes), electrical impedance, and forward angle light scatter.

New automated analyzers have evolved out of this same principle like the UF-50 and Bacsys. The UF-50 is a smaller version of the UF-100 for lower throughput while the Bacsys is a dedicated instrument for use in the microbiological screening of urine and other human body fluids. It quantifies both bacteria and leukocytes based on fluorescence flowcytometry using a proprietary fluorescence polymethine dye which is excited by a red semi-conductor laser (11-13). This laser has a lower operating cost than the one used in the previous versions.

Meanwhile, an improved version of the IRIS, the Iris iQ200 Automated Urine Microscopy Analyzer (Iris Diagnostics, USA), also based on imaging technology using a video camera and strobe lamp to detect and sort particles based on predetermined particle dimensions, was developed and is gaining use in routine clinical laboratories (14). It disposes of a neural network to improve categorization of the urinary particles. An extra advantage of this system compared to others is its mechanic, not only software-based, coupling with an automated dipstick reading device.

The greatest contribution of both concepts is the improved precision and sample throughput compared with manual microscopy.

Specific tubular proteins in urinalysis

References (15-25) deal with this paragraph.

As with electrophoresis, quantitative analysis of urine marker proteins has been successfully applied to detect and differentiate nephrological and urological disorders. These microproteins are not only tools to non-invasive early detection of renal abnormalities, whilst their purpose usually is to locate the source of the proteinuria or hematuria.

Traditionally, total protein and albumin were measured but now also other proteins can be helpful in diagnosing urinary tract disease.

For evaluation of the glomerular function, IgG and alpha 2-macroglobulin can be used. To assess the tubular (dys)function, A1M, beta 2-microglobulin, retinol binding protein, Clara cell protein and N-acetyl-beta-D-glucosaminidase are available of which the first three are mostly available in dedicated laboratories. Not all these analytes are equally fit to be used in routine clinical practice: problems may exist concerning their stability in urine (beta 2-microglobulin) or availability of routine assays (retinol binding protein, N-acetyl-beta-D-glucosaminidase). Everaert et al. showed A1M to be useful in the detection of renal tubular damage in patients with outflow disease of the upper tract and to be diagnostic for vesico-ureteral reflux (VUR).

Most important reasons for selecting A1M in our studies are the combination of its stability in human urine ex vivo together with the availability of easy and automated test kits (especially on a routine turbidimetric device such as a Modular P, Roche Diagnostics, Mannheim, Germany) allowing it to be introduced in a routine clinical laboratory.

Expert systems

The general framework of the routine clinical laboratory of the early 21st century is characterized by an increasing integration of different techniques on one platform e.g. chemistry and immunochemistry. Furthermore, since routine clinical laboratories are confronted with a growing complexity of diagnostic tests, an increased workload, stringent laboratory accreditation demanding increasing standardisation, continuous shortening of turn-around-time and budget restrictions, the introduction of artificial intelligence by

means of expert systems has gained an important place in the automation process to meet these demands.

In this view, it seemed logical to combine data in urinalysis gathered by different techniques but from the same sample since both techniques used (flow cytometry and test strip analysis) have different methodological pitfalls inherent to the method. In 1999, Langlois et al (21) developed a rule-based expert system that performs a cross-check of flow cytometric data (UF-100) with results obtained by semi-quantitative dipstick testing allowing a clinically acceptable sieving system to reduce the workload of microscopic sediment urinalysis. The current use of this system in our laboratory and a short description is explained in more detail in chapter 3.

By using knowledge based expert systems, expert knowledge can improve the information obtained from urine analysis. Professor W. Guder and co-workers elaborated a Urine Protein Expert System (UPES) based on a basic set of urinary proteins and a test strip screening (26-31). It was successfully integrated and evaluated to support clinical diagnosis showing that non-invasive urine protein differentiation may be a useful diagnostic strategy in nephrology. The multivariate evaluation of the excretion pattern allows differentiation of prerenal from glomerular, tubular, and postrenal causes of proteinuria and hematuria.

This system combines a rule-based pattern definition database with implemented logical and mathematical algorithms. The interpretative text covers not only underlying kidney disorders but also comments on preanalytical and analytical problems.

Expert systems are not a privilege of urinalysis alone. Also for enzyme electrophoresis such as for amylase and alkaline phosphatise, expert systems have been developed (32). These systems combine the data from the specimen analysis and data covering patient characteristics like age and sex featuring a bidirectional link with the Laboratory Information System (LIS). The software then produces a report that can be communicated to the clinician to support the interpretation and to suggest possible work-outs.

CHAPTER 2

Outlines

&

aims

CHAPTER 2: Outlines and aims

Despite the older view that urinalysis is not the most exciting and dynamic area in clinical laboratory medicine and that it is sometimes being regarded as an ancient, non-evolving technique, improvements are still ongoing and are more than necessary.
Urinalysis always has been a cumbersome, time-consuming technique subject to great inter-operator variability. The first, not completely successful, attempt to automate urine microscopy was based on photographic capturing techniques (UA-1000 and UA-2000 from Sysmex Corporation, Kobe, Japan; Yellow IRIS from Iris Diagnostics, Chatsworth, USA). More recent developments of automated techniques comprise urinary flow cytometry (UF-100, UF-50 and Bacsys; Sysmex, Japan) and automated urine microscopy readers (IQ200, Iris Diagnostics, Chatsworth, USA) introducing a faster and much more refined and standardized technique in this cumbersome field. Inter-observer variance rates dropped dramatically while sample throughput rose significantly.
In our laboratory, most experience is gained regarding the application of the UF-100 in a routine clinical laboratory setting. Certain pitfalls exist in using this technique: yeasts can be counted as RBC, extreme pH conditions can destroy some brittle urinary formed elements like casts, etc. So it was a first logical step to combine flow cytometric data with urinary test strip results to overcome some of the weaknesses of both techniques. In 1999, Langlois et al. implemented this practice in such an expert system (21).
However, as automated test strip readers, which were developed much earlier than flow cytometry, reported semi-quantitative data, there was an imbalance of the quality of the obtained data being quantitative on one hand (flow cytometry) and ordinal scaled data on the other hand (test strip readers). With the newer test strip readers, quantitative reflectance data are available to the user. We were interested in the quality of these data in order to integrate them in an improved expert system.
Before the availability of fingerprick (skin puncture) tests, urine test strips were also used to monitor diabetics as a warning for imminent diabetic ketoacidosis (DKA). With fingerprick tests being available, this application of urine test strips is obsolete but other problems arise in specific patient groups. For diabetics treated with an insulin infusion pump, monitoring of acetoacetate (AcAc) and 3-hydroxybutyrate (3HB) after an episode of DKA is of clinical importance. Commercial test strips are available for monitoring blood 3HB but these strips are expensive. We investigated the possible use of urine test strips in this setting.

Standard urinary test strips can only measure AcAc and not 3HB. Using a specific sample pre-treatment we were able to measure both AcAc and 3HB and applied this technique in this specific setting.

In a routine clinical laboratory, other body fluids with expected low cell counts are investigated, such as cerebrospinal fluid and peritoneal dialysis fluid from continuous ambulatory peritoneal dialysis (CAPD). The presence of a low number of cells, as found in urine, makes it logical to investigate the usability of a urinary flow cytometer for these samples. Van Acker et al. (33) already described their experience with investigating cerebrospinal fluid using this technique. Later on, Aps et al. applied it to saliva (34) and Muylaert et al evaluated it for sperm counting (35). We investigated the possible use and pitfalls of using a urine flow cytometer in case of CAPD-fluid.

Specific proteins play an important role in evaluating urinary tract disease and expert systems, like UPES developed by Professor Guder and Dr. Ivandic, can provide nephrological diagnoses based on specific urinary protein analysis (28;29).
Specifically A1M seems to be an interesting protein given its stability in a broad urinary pH-range and the availability of convenient automated assays. The most important drawback at the time being is its lack in standardisation. After a literature search, we evaluated whether several possible ratios based on A1M and data from test strip and flow cytometric analysis would be of assistance in the evaluation of urological and renal conditions.

Main goal

The main goal of this thesis was to explore the possibilities of the available state-of-the-art automated urinalysis techniques in a routine laboratory setting, to expand their use in clinical and laboratory practice, and to improve the diagnostic possibilities by combining the information obtained by these individual techniques.

Specific aims

- To investigate the possible advantage of quantitative urine strip analysis compared to the routinely used semi-quantitative reporting.
- The possible use of quantitative test strip reading in a clinical setting of diabetic patients

treated with insulin pumps.

- The application of urine flow cytometry in other body fluids with low cell counts especially peritoneal dialysis fluid.
- Combining the information provided by flow cytometry, test strips and specific urinary proteins for use in diagnosis and monitoring urological and nephrological disease.

CHAPTER 3

Application

of

test strips

and

alpha 1-microglobulin

in

urinalysis

CHAPTER 3: Application of test strips and alpha 1-microglobulin in urinalysis

This chapter covers the chemical principles handled in this thesis.
Chapters 3A (Quantitative evaluation of urinalysis test strips) and 3B (Quantitative measurements of ketone bodies in urine using reflectometry) handle about specific applications of urinary test strips. Chapter 3C (Alpha 1-microglobulin: clinical laboratory aspects and applications) contains a review about A1M, in preparation of the investigation about combining information from different techniques (flow cytometry, proteins and dipstick) to diagnose urinary tract disorders which is covered further on.

Present situation in the laboratory of Clinical Biology at Ghent University

The standard operating procedure for urine microscopy and test strip analysis in our laboratory consists of the combined use of a UF-100 flow cytometer (Sysmex, Kobe, Japan) and a test strip reader (one Urisys 2400 and one Miditron; Roche Diagnostics, Mannheim, Germany). In the ideal case, every sample is presented first to one of the test strip readers followed by flow cytometric analysis. The two instruments are linked by software that compares the results of both analysers and, on the basis of a set of rules compiled in an expert system, it produces a print-out of the samples and parameters that should be re-investigated by a trained laboratory technician. For example, high RBC and WBC counts raise flags on the UF-100 screen to review the specimen under a microscope. However, when UF-100 RBC and WBC data are concordant with dipstick hemoglobin and leukocyte esterase reactions, there would be no need for additional microscopic confirmation.
Remarks are also reported (e.g. old sample?, Proteus?, Myoglobunuria?, Sterile pyuria?, Hyaline casts?, Pathological casts?, Total number>250000, Protein, Hematuria?, Casts are unstable in alkaline urine).
The operator only checks and corrects the parameters indicated. After re-analysis is completed, the reviewed results together with the remarks are transmitted to the LIS to be reported to the physician.

Urine test strip reading: instrument description

The classical test strip is read a certain time after dipping in urine with the help of the ambient light. This, of course, is very subjective and user dependent since the brightness of the light differs and the time after reading is not fixed.

To standardize this process automated test strip readers (reflectometers) have been developed that work based on the principle of reflectometry in which a standardized light source (usually a LED) is used and the percent reflectance is measured. 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.

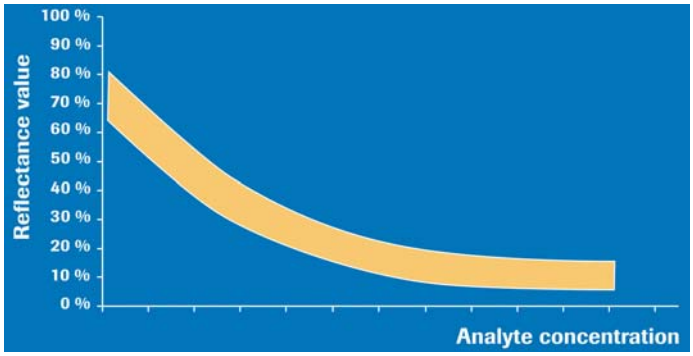

Figure 1: the relation between analyte concentration and % reflectance

Despite these originally quantitative results, until recently it was common practice to express results as ordinal scaled (normal, negative, positive, 0, +, ++, etc.). With the latest generation of test strip readers it is possible to obtain the quantitative reflectance data for all of the classically used test pads: leukocyte esterase, nitrite, protein, glucose, ketones, urobilinogen, bilirubin, hemoglobin/myoglobin, and pH.

The automated reader used in our studies was the URISYS 2400 (Roche, Germany). It measures the usual analytes as described above while 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 test strip. The reflectance data can be downloaded or forwarded to linked PC's.

It is this instrument that we evaluated in the first study presented in chapter 3A (Quantitative evaluation of urinalysis test strips) by comparing it with validated

biochemistry methods. It was our intent to investigate the possible added value and the analytical performance of the originally quantitative reflectance data.

Ketone bodies and diabetic ketoacidosis (DKA)

Low concentrations of ketone bodies are always found in the blood and urine of a healthy reference population. This can be explained by the fact that ketones are a primary source of energy for some organs and a secondary or alternate source for others. Three ketone bodies exist: 3HB, AcAc and acetone of which 3HB and AcAc are the most important ones. Acetone, the least abundant ketone body, is eliminated primarily through respiration causing the characteristic ‘‘pear-drop’’ smell present in DKA.

In response to certain stresses or in various medical conditions, ketone body levels rise in both blood and urine. This is of particular interest in diabetics who are much more vulnerable for metabolic imbalance, possibly leading to DKA. The objective of routine ketone monitoring, especially of 3HB and AcAc, is to detect and thus prevent DKA.
The American Diabetes Association recommends that anyone with diabetes performs urinary tests for ketones during times of illness or stress, during pregnancy, when blood glucose levels of 17 mmol/L persist, or whenever symptoms of ketoacidosis are observed. The presence of precipitating factors that contribute to the development of DKA would also indicate that ketones should be monitored. While diabetes type 1 patients are at particular risk, ketoacidosis is increasingly recognized as a problem associated with type 2 patients as well, especially those who are insulin-dependent.
To diabetics treated with an insulin infusion pump, monitoring of AcAc and 3HB is of clinical importance to reduce the misdiagnosis of DKA (e.g., due to undetected leakage of the infusion system, infection, mismanagement of the infusion pump).
In DKA, the ratio of 3HB to AcAc increases from 1:1 to as much as 5:1 making 3HB the most important ketone body to monitor in this situation.
When acidosis resolves with treatment, 3HB is oxidized to AcAc, returning the ratio to baseline conditions.

Classically, the nitroprusside reaction on urine test strips (Ketostix, Acetest) has been used to carry out semi-quantitative assays of urinary ketones. A drawback of this method is that it mainly measures AcAc. The presence of ketone bodies in urine at concentrations detectable by strips has long been recognized as a symptom of DKA. However, since

AcAc is measured semi-quantitatively and the nitroprusside reaction reacts weakly with acetone and does not register the presence of 3HB, this technique has limited value in judging DKA. When 3HB is the only ketone present, which can occur occasionally, the colour reaction underestimates the magnitude of ketoacidosis.
Moreover, when the acidosis is resolved by treatment, AcAc levels remain high due to the oxidation of 3HB. When using urine test strips, this may lead to the wrong impression that ketosis is not improving.

Since 3-HB is the major ketone body in DKA, the monitoring of AcAc may not be clinically important at all, or may be misleading. Nowadays, an expensive 3HB assay can be used on a handheld sensor (Optium meter; Medisense/Abbott, Abingdon, UK) to measure this ketone body using blood from a fingerprick test.
However, compared to the traditional nitroprusside reaction on urine test strips, an accurate quantitative assessment of all ketone bodies in urine could provide a much more valuable insight in determining current metabolic status and treatment strategy. We evaluated the possibility to use urine test strips for this purpose. Therefore it was necessary to first develop a workaround for the specificity of the ketone test pad for AcAc. After a technical evaluation, samples were measured for AcAc and 3HB with the urine test strips and a validated routine chemistry method (chapter 3B: Quantitative measurements of ketone bodies in urine using reflectometry).

Alpha 1-microglobulin (A1M)

The evaluation of renal tubular function is an important issue in clinical laboratory medicine. Detection of urinary microproteins can help to detect renal abnormalities at an early stage and differentiate the various forms of renal and urological pathology by less invasive techniques. Several markers exist but the stable microprotein A1M, also called protein HC, is one of the most interesting ones due to its stability.
We first made a round-up of the existing information of this protein so we could possibly use this in a second stage for diagnosing urological and nephrological diseases. Recent peer-reviewed literature was systematically checked until November 2003. Our findings were pooled and presented such that it can be used in the clinical laboratory (chapter 3C: Alpha 1-microglobulin: clinical laboratory aspects and applications).

Chapter 3 A

Quantitative evaluation of urinalysis test strips

Joris Penders, Tom Fiers, and Joris R. Delanghe

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Automation and Analytical Techniques

Quantitative Evaluation of Urinalysis Test Strips

Joris Penders, Tom Fiers, and Joris R. Delanghe*

Background: **Urine test strip results are generally reported in categories (i.e., ordinal scaled), but automated strip readers are now available that can report quantitative data. We investigated the possible use of these meters to complement flow cytometry of urine and compared reflectance readings with quantitative determinations of urinary glucose and microalbumin.**

Methods: **We compared URISYS 2400 (Roche) quantitative reflectance data with data from the UF-100 (Sysmex) and biochemical data for 436 nonpathologic and pathologic urine samples.**

Results: **Reproducibility of the reflectance signal was good for high- and low-concentration urine pools for protein (0.8% and 0.9% and 1.5% and 2.2% within and between runs, respectively), leukocyte esterase (1.1% and 1.0%; 5.1% and 1.2%), hemoglobin (1.7% and 1.1%; 8.9% and 1.1%) and glucose (2.1% and 0.5%; 6.5% and 2.3%). Fair agreement was obtained between UF-100 and test strip reflectance data for erythrocytes and hemoglobin ($r = -0.680$) and leukocytes and leukocyte esterase ($r = -0.688$). Higher correlations were observed for biochemical and test strip data comparing protein and albumin ($r = -0.825$) and glucose data ($r = -0.851$). The lower limits of detection for erythrocytes and leukocytes were 8×10^6/L and 19×10^6/L, respectively. The protein test (n = 220) detected 86% (95% confidence interval, 78–92%) of samples with <30 mg/L albumin with a specificity of 84% (95% confidence interval, 76–91%).**

Conclusions: **In urine test strip analysis, quantitative hemoglobin and leukocyte esterase reflectance data are complementary with flow cytometric results and glucose and albumin results.**

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 *(1)*. The reproducibility of (semi)automated readings is at least as good as visual readings *(2)*, but most authors find the analytical, clinical, and labor cost-saving advantages of (semi)automated vs visual reading to be obvious *(3)*.

Recently, the URISYS 2400 automated urine test strip analyzer (Roche Diagnostics) was introduced. This instrument offers the possibility to obtain reflectance readings. Test results, therefore, no longer need to be expressed in an ordinal scale. Access to the instrument's raw data theoretically allows a higher analytical sensitivity for several analytes. Because microalbuminuria is generally regarded as an excellent marker for assessing early renal damage in common conditions such as diabetes and hypertension *(4–7)* and as an early predictor of preeclampsia during pregnancy *(8)*, the availability of highly sensitive test strip readers opens interesting perspectives for assessing this phenomenon.

Flow cytometry has been introduced for urinalysis *(9, 10)* to obtain quantitative data on urinary particles. The imprecision of urinary flow cytometry is far less than that of conventional urinary microscopy, but in some cases urinary flow cytometry reports erroneous results because of analytical interferences (e.g., calcium carbonate crystals falsely increase erythrocyte counts; confusion between yeast cells and erythrocytes). Langlois et al. *(11)* reported disagreement in erythrocyte counts between the UF-100 and the hemoglobin test strip reaction in 6.5% of cases. Of course, the frequency of erroneous results depends on the proportion of pathologic samples and on the preanalytical handling of samples. Combining diagnostic information provided by urinary flow cytometry and more quantitative test strip analysis therefore offers a theoretical basis for the development of diagnostic expert systems *(11, 12)*.

In this study, we wanted to investigate the performance of quantitative urinary test strip analysis. In particular, we wanted to compare the reflectance readings of the protein test field with immunochemical microalbumin determinations. We also wanted to explore the possibilities of combining the two novel methods, particularly in the analysis of erythrocytes, leukocytes, and glucose, which are of major clinical importance.

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Materials and Methods

PATIENTS AND SAMPLES

We studied 436 freshly collected urine samples submitted to our routine laboratory for diagnostic urinalysis. All samples were completely processed within 2–4 h after arrival. Test strip urinalysis was carried out before flow cytometry analysis (Sysmex UF-100; TOA Medical Electronics), using URISYS strips on a URISYS 2400 analyzer (Roche Diagnostics). Combur 10-Test M strips on a Miditron automated reflectance photometer (Roche) *(13, 14)*, used in our routine laboratory, were used in parallel as a control. The strips include reagent pads for ordinal scale reporting of relative density, pH, leukocyte esterase, nitrite, protein, glucose, ketones, urobilinogen, bilirubin, and hemoglobin/myoglobin.

Day-to-day imprecision was assessed with control material: Liquichek Urinalysis Control Levels 1 and 2 (Bio-Rad). This is stable for 30 days when stored tightly capped at 2–8 °C.

URINARY FLOW CYTOMETRY

The Sysmex UF-100 is a urinary flow cytometer-based walkaway instrument that performs automated microscopic analysis. It has been extensively evaluated for urinalysis *(9–11, 15)* as well as for cerebrospinal fluid analysis *(16)*. The principle is based on argon laser flow cytometry. The UF-100 measures the conductivity and categorizes the particles on the basis of their shape, size, volume, and staining characteristics. The results are displayed in scattergrams, histograms, and as counts per microliter as well as counts per high-power field. The UF-100 automatically detects and counts red blood cells (RBCs), white blood cells (WBCs), bacteria, yeast cells, crystals, epithelial cells, small round cells, sperm cells, and casts. Particles that cannot be classified in one of the former categories are counted as "other cells".

URISYS 2400

Urine test strip analysis was performed with the automated URISYS 2400. These test strips are the same as used in the Miditron but are supplied in a cassette holding 400 test strips for leukocyte esterase, nitrite, protein, glucose, ketones, urobilinogen, bilirubin, hemoglobin/myoglobin, and pH.

The intensity of the reaction color 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 color 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 color is rated with a specific algorithm against the blank pad on the test strip. Data are expressed in an ordinal scale (as "normal", "negative", "positive", or as nominal concentrations) on the reports, but (quantitative) reflectance data can be downloaded to floppy disks.

BIOCHEMICAL INVESTIGATIONS

Albumin in urine was measured immunonephelometrically on 220 randomly selected samples with use of commercially available Behring antibodies on a Behring Nephelometer II analyzer (Dade Behring) standardized against the widely accepted WHO/College of American Pathologists Certified Reference Material 470. Glucose was measured by a hexokinase method standardized against Standard Reference Material 917a and Standard Reference Material 965 (n = 85), and total protein by a pyrogallol red method *(17)* (n = 129) with Standard Reference Material 917a as a standard and commercially available reagents for both measurement procedures (Roche) on a Modular P system (Roche).

STATISTICS

P values <0.05 were considered significant. Agreement between automated flow cytometry and test strip data was evaluated by Spearman rank analysis. Multiple regression analysis was used to investigate a model relating leukocyte esterase and hemoglobin field reflectance. The lower limit of detection *(18)* was calculated as the mean value − 3 SD for a blank sample. Diagnostic accuracy was assessed by ROC analysis using commercially available statistical software (MedCalc®).

Results

DILUTION AND pH

Alkalinization of urine gives rise to erroneous results in particle counting. When monitoring our samples, we found a median pH of 6.5 (95% interval, 5–8). Hence, extremely alkaline urine samples were not present in the samples we investigated. Dilution was also monitored with a range of specific gravity of 1.005–1.033 and a median result of 1.017.

REPRODUCIBILITY

The within- and between-run CVs for protein, leukocyte esterase, hemoglobin, and glucose are summarized in Table 1.

COMPARISON OF PROTEIN REFLECTANCE RESULTS, ALBUMIN, AND PROTEINURIA

We found a close correlation between the log-transformed albumin results (x) and the test strip reflectance results (y): y (%) = 68.2 − 9.26[log$x$ (mg/L)]; Spearman $r = -0.825$; $P < 0.001$; Fig. 1. Two discrepancies (0.9%) were found in which the test strips on both automated strip readers overestimated the urinary albumin concentration by >10-fold.

Similarly, protein reflectance data and total protein measurements correlated well (Spearman $r = -0.921$; $P < 0.001$), which is expected as long as the major protein is albumin. The lower limit of detection was 25 mg/L (CV =

Table 1. Reproducibility of protein, leukocyte esterase, hemoglobin, and glucose on the URISYS 2400 analyzer.

	Protein (albumin)			WBCs			RBCs			Glucose		
Urine pool	CV, %	Mean reflectance, %	Mean concentration, mg/L	CV, %	Mean reflectance, %	Mean count, 10^6/L	CV, %	Mean reflectance, %	Mean count, 10^6/L	CV, %	Mean reflectance, %	Mean concentration, mmol/L
Within-run												
High concentration	0.8	52.0	56	1.1	48.4	196	1.7	14.7	1874	2.1	30.6	16
Low concentration	0.9	59.5	9	1.0	61.5	27	1.1	63.0	6	0.5	69.9	0.15
Between-run												
High concentration	1.5	38.3	1694	5.1	22.5	9419	8.9	18.9	1139	6.5	26.6	26
Low concentration	2.2	60.2	7	1.2	64.1	19	1.1	65	5	2.3	66.1	0.23

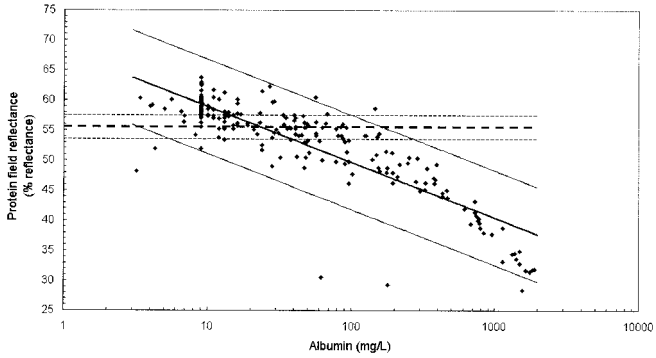

Fig. 1. Correlation between protein (albumin) results obtained by quantitative test strip analysis (*y*) and albumin (*x*; Behring BN II Nephelometer; n = 220).

Protein field reflectance (*y*; %) = 68.2 − 9.26 log(albumin; mg/L); Spearman $r = -0.825$; $P < 0.001$). The *two outer solid lines* represent the 95% prediction interval around the *regression line*. The *dashed lines* represent the lower limit of detection (− − −) with the 2 SD limits (- - -).

2.2%) *(18)*. On the basis of ROC analysis, diagnostic sensitivity was 86% (95% confidence interval, 78–92%) and specificity was 84% (95% confidence interval, 76–91%) for a reflectance value of 55.6% when the nephelometric assay was used as the comparison method at a cutoff of 30 mg/L (n = 220; 113 positive and 107 negative cases, respectively).

COMPARISON OF FLOW CYTOMETRIC RBC AND HEMOGLOBIN REFLECTANCE RESULTS

Agreement was fair between the flow cytometric RBC data (*x*) and the URISYS 2400 hemoglobin reflectance measurements (*y*) for counts above the upper reference limit (25×10^6 RBC/L on UF-100). The following regression equation was obtained: y (%) = 78.2 −19.4 log$x$ (10^6 cells/L); Spearman $r = -0.680$; $P < 0.001$; Fig. 2. A lower limit of detection of 8×10^6 cells/L (CV = 1.2%) was calculated.

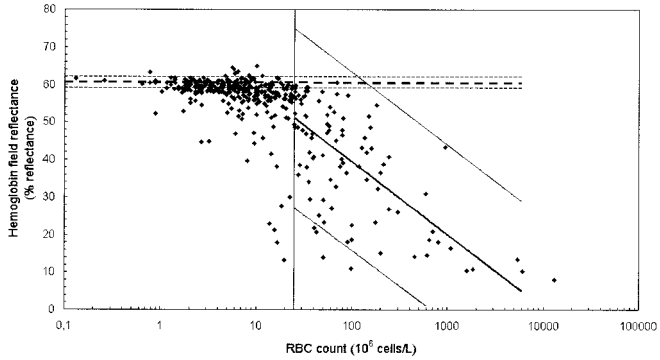

Fig. 2. Correlation between RBC counts (*x*; flow cytometry) and test strip hemoglobin concentration (*y*) for RBC counts >25×10^6 cells/L (n = 96).

Hemoglobin reflectance (*y*; %) = 78.2 − 19.4 log(RBC count; 10^6 cells/L); (Spearman $r = -0.680$; $P < 0.001$. The two *outer solid lines* represent the 95% prediction interval around the *regression line*. The *dashed lines* represent the lower limit of detection (− − −) with the 2 SD limits (- - -).

Because RBCs tend to lyse in urine, we investigated the linearity of the hemoglobin test strip pad. The UF-100 flow cytometer automatically determines the conductivity, so we performed multiple regression analysis on the study population, calculated in a regression model the hemoglobin reflectance vs RBCs and conductivity, and found that the *t*-value (as expected) for RBCs was −8.442 ($P < 0.0001$). In contrast, conductivity showed a *t*-value of only 1.477 ($P = 0.141$). Additional dilution experiments were carried out. The ratio of hemoglobin reflectance to RBC count was constant when osmolality was >190 mosmol/L.

comparison of flow cytometric wbc and leukocyte esterase reflectance results

The correlation between WBC data (x) and leukocyte esterase measurements (y) is shown in Fig. 3. When urinary WBC counts were higher than the upper reference limit (25×10^6 cells/L), the following regression equation was obtained: y (%) = 83.7 −15.4 log$x$ (10^6 cells/L); Spearman $r = -0.688$; $P < 0.001$. For WBCs, we found a lower limit of detection of 19×10^6 cells/L (CV = 1.2%).

Multiple regression analysis on the study population showed no effect of conductivity on test strip leukocyte esterase.

comparison of the glucose measurements

We also found good agreement between the hexokinase-based glucose method (x) and glucose test strip reflectance reading (y): y (%) = 53.9 − 19.3 log$x$ (mmol/L); Spearman $r = -0.851$; $P < 0.001$. Fig. 4 depicts the correlation between both measurement methods and the lower limit of detection of 0.17 mmol/L (CV = 1.3%) with the 2 SD limits.

Discussion

The change from urine microscopy to urinary flow cytometry has been accompanied by a significant decrease in imprecision *(15)*. Because urinalysis test strips are often used for checking urinary flow cytometry data *(11)*, there is a need for a more quantitative evaluation of urinalysis test strips. In this study, we compared the URISYS 2400 automated strip reader with the Sysmex UF-100 flow cytometer to evaluate the possible value of quantitative test strip data in a urinary expert system.

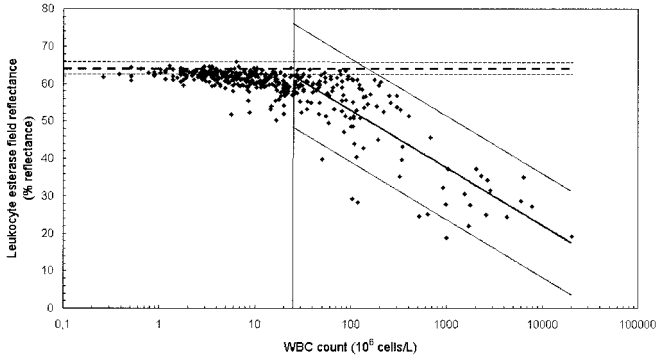

Fig. 3. Regression equation for urinary WBC counts above the upper reference limit (25×10^6 cells/L; n = 132).

Leukocyte esterase reflectance (y; %) = 83.7 − 15.4 log(WBC count; 10^6 cells/L); Spearman $r = -0.688$; $P < 0.001$. The two *outer solid lines* represent the 95% prediction interval around the *regression line*. The *dashed lines* represent the lower limit of detection (− − −) with the 2 SD limits (- - -).

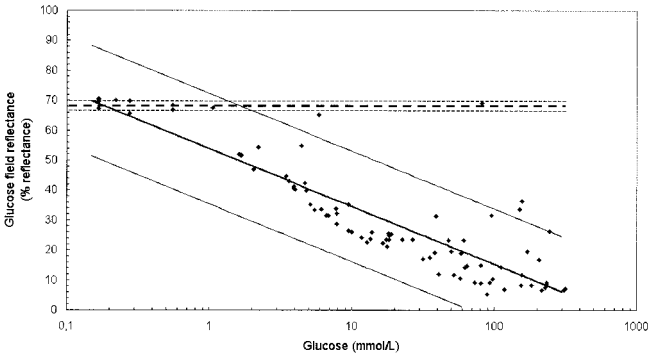

Fig. 4. Correlation between glucose measurements (glucose concentration different from 0) by the hexokinase-based method and glucose test strip reading (n = 85).

Glucose reflectance (y; %) = 53.9 − 19.3 log(glucose; mmol/L); Spearman $r = -0.851$; $P < 0.001$). The two *outer solid lines* represent the 95% prediction interval around the *regression line*. The *dashed lines* represent the lower limit of detection (− − −) with the 2 SD limits (- - -).

The turnaround time was short enough (2–4 h) not to affect the readings: time intervals can influence results of automated test strip analysis, especially leukocyte and erythrocyte ratings *(19)*.

The detection limit of the URISYS 2400 protein assay was 25 mg/L if restricted to albumin alone and total protein was not considered. Because microalbuminuria is defined as excretion of 30–300 mg of albumin/24 h (20–200 μg/min, or 30–300 μg/mg of creatinine) *(20)*, the protein field result allows identification of microalbuminuria cases in contrast to the classic reading and reporting of the strips, which can detect only albumin concentrations 150–200 mg/L or higher *(21, 22)*. This implies that the test has the potential to offer a screening for microalbuminuria without increased cost, hence coming closer to the "urine test strip of the future" *(23)*. This could improve level 1 urinary screening *(12)*: it offers an improved, fast, reliable method that is easy to handle and usable in primary-care laboratories. It could be of special interest in patients with undiagnosed diabetes or hypertension in whom microalbuminuria is regarded as an excellent marker for assessing early renal damage *(4–7)*, particularly because many patients with non-insulin-dependent diabetes mellitus are asymptomatic and their diabetic state remains undiagnosed for years *(24)*. It must be noted, however, that the sensitivity (86%) may be inadequate for patients with known diabetes in whom physicians do not want to miss microalbuminuria in their annual testing. Moreover, a 16% false-positive rate may be unacceptable for screening.

Because microalbuminuria is characterized by large intraindividual variability, an ordinal-scale answer might

be misleading. It therefore is advantageous to have a better estimation of the true concentration.

As is the case for test strips in general, the method is based on the so-called "indicator error" principle, in which proton exchange between the indicator on the strip and the proteins in the solution produces a color change from yellow to green-blue. Of all the diagnostically relevant urinary markers, only albumin and transferrin accept protons well, so the potential error of missing Bence Jones proteinuria remains when test strip screening strategies are used in proteinuria *(21)*, although many cases presenting with Bence Jones or tubular proteinuria show microalbuminuria *(23)*.

In recent studies, the correlation (r) between UFC WBCs and WBCs by counting chamber was 0.93–0.98, and that of UFC RBCs and RBCs by counting chamber was 0.83–0.89 *(10, 25, 26)*. In our study correlating test strip data with urinary flow results, r was −0.69 between the flow cytometric WBC count and the leukocyte esterase reaction, although the presence of esterase inhibitors in urine and severe proteinuria might negatively affect test results for leukocyte esterase *(1, 27)*. No effect of conductivity on the leukocyte esterase field was noted. Test strip measurements had reasonable lower limits of detection for WBC (19×10^6/L).

In this study, the urinary hemoglobin concentration did not agree well with the RBC count obtained by flow cytometry. The hemoglobin measurement is based on the peroxidase principle. It is known that reducing substances (e.g., ascorbic acid) may lower the signal, whereas oxidizing substances may have a positive effect on measured hemoglobin concentration. Various low- and high-molecular mass inhibitors have been found in urine *(28, 29)*. The presence of haptoglobin in urine enhances the peroxidase activity of hemoglobin *(29, 30)*. Bacterial peroxidases can also contribute to total peroxidase activity in urine *(23)*. On the other hand, the quantitative evaluation of test strips may help to eliminate analytical errors in RBC counting attributable to the presence of yeast cells or large amounts of calcium carbonate crystals *(11, 15)*. As shown by our multiple regression model and dilution experiments, the hemoglobin field reflectance test is not influenced by dilution effects. As is the case for WBCs, the lower limit of detection for RBCs is acceptable (8×10^6/L).

The correlation coefficient for the glucose signal with the routine hexokinase-based method was −0.851. The presence of ascorbate oxidase on the glucose test field prevents interference by ascorbic acid.

Because of the procedure for applying the urine to the test strips on the URISYS 2400, improper dipping is no longer a problem, nor is confusion about sample identification or urine contamination caused by dipping the strip in the tube, which potentially leads to interferences with chromatographic methods *(31)*.

In conclusion, quantitative urine test strip analysis provides reliable data on WBCs, RBCs, glucose, and albumin. This offers several possibilities: (*a*) The sensitivity for albumin may allow affordable screening for microalbuminuria, particularly in patients with undiagnosed renal damage. To fully explore the possibilities of the albumin test pad in first-line diagnosis, a formal study should be performed. (*b*) In addition, hemoglobin and leukocyte esterase reflectance data are useful for verifying flow cytometric data on RBCs and WBCs. This leads to improved elimination of occasional errors in the WBC and RBC counting channels of the flow cytometer *(11)*.

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Chapter 3 B

Quantitative measurement of ketone bodies in urine using reflectometry

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Quantitative measurement of ketone bodies in urine using reflectometry

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Abstract

Background: Recently, automated urine test strip readers became available that can report quantitative data. We explored the possibility of measuring all ketone bodies (acetone, acetoacetate, 3-hydroxybutyrate) in urine with these test strips. Monitoring urinary ketone concentrations could offer the advantages of measuring higher values (due to the low renal thresholds) and being less sensitive to fluctuations.

Methods: We evaluated URISYS 2400 (Roche) quantitative reflectance data for the ketone reflectance field and compared it with biochemical data from urine samples. Using an easy sample pre-treatment with 3-hydroxybutyrate dehydrogenase, we were able to assay 3-hydroxybutyrate as well, which normally does not react on urine test strips.

Results: Within- and between-run reproducibility of the reflectance signal for high- and low-concentration urine pools was 11.0–3.6% and 11.0–5.8% for acetoacetate, 8.2–9.2% and 10.4–16.1% for acetone, and 5.1–3.0% and 5.6–3.5% for 3-hydroxybutyrate, respectively. The lower limit of detection for acetoacetate was 0.13 mmol/L (CV = 3.6%). Fair agreement was obtained between test strip data for ketones and colorimetrically determined acetoacetate values ($r = 0.90$).

Conclusions: In urine test strip analysis, quantitative ketone reflectance data allow a simple and fast analysis, offering affordable screening for the detection of ketone body production in diabetes, especially in emergency settings.

Keywords: acetoacetate; diabetic ketosis; 3-hydroxybutyrate; reagent strip; urinalysis.

Introduction

In diabetes monitoring, there is growing interest in monitoring the production of ketone bodies. In particular, for diabetics treated with an insulin infusion pump, monitoring of acetoacetate (AcAc) and 3-hydroxybutyrate (3HB) is of clinical importance (1–3).

As a primary source of energy for some organs and a secondary or alternate source for others, low concentrations of ketone bodies are always found in the blood and urine of a healthy reference population. However, in response to certain stresses or in various medical conditions, ketone body levels rise in both blood and urine. The objective of routine ketone monitoring, especially of 3HB and AcAc, is to detect and thus prevent diabetic ketoacidosis (DKA). Acetone, the third and least abundant ketone body, is responsible for the characteristic ''pear-drop'' smell present in DKA, but does not contribute to acidosis (4, 5).

Classically, the nitroprusside reaction (Ketostix, Acetest) has been used to carry out semi-quantitative assays of urinary ketones (6), mainly measuring AcAc. The presence of ketone bodies in urine at concentrations detectable by strips has long been recognized as a symptom of DKA (6). However, since AcAc is measured semi-quantitatively and the nitroprusside reaction reacts weakly with acetone and does not register the presence of 3HB, this technique has limited value in judging DKA (2, 7). When 3HB is the only ketone present, which can occur occasionally, the color reaction underestimates the magnitude of ketoacidosis (4, 5).

In DKA, the ratio of 3HB to AcAc increases from 1:1 to as much as 5:1. When acidosis resolves with treatment, 3HB is oxidized to AcAc. Under these circumstances, urine tests may give the misleading impression that ketosis is not improving (4). Nowadays, an expensive 3HB assay can be used on a handheld sensor (Optium meter; Medisense/Abbott, Abingdon, UK) to measure this ketone body using blood from a fingerprick test (8).

However, compared to the traditional nitroprusside reaction on urine test strips, an accurate quantitative assessment of all ketone bodies in urine could provide a much more valuable insight in determining current metabolic status and treatment strategy. The quantitative monitoring of ketone bodies could improve evaluation of the therapeutic progress in ketoacidosis in the clinical setting.

The value of test strip urinalysis has been thoroughly proven (9). The reproducibility of (semi-)automated reading is at least as good as visual reading (10), but most authors find the analytical, clinical and labor-cost saving advantages of (semi-)automated vs. visual reading to be obvious (11). Some years ago, the URISYS 2400 automated urine test strip analyzer (Roche Diagnostics, Mannheim, Germany) was introduced. This instrument offers the possibility to obtain reflectance readings. Test results therefore no longer

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need to be expressed on an ordinal scale. Access to the instrument's raw data theoretically allows higher analytical sensitivity (12).

In this study, we wanted to investigate the performance of quantitative measurements of urinary ketones using reflectometry. Results were compared with those of photometric methods for the determination of AcAc and 3HB. Using a sample pretreatment with 3-hydroxybutyrate dehydrogenase (EC 1.1.1.30), the possibility of analyzing urinary 3HB using quantitative reflectometry was studied. Furthermore, we wanted to explore the possibilities of quantitative ketone measurements in a clinical setting.

Materials and methods

Test strip analysis

Test strip urinalysis was carried out using URISYS strips on a URISYS 2400 analyzer (Roche Diagnostics) (12). The strips include reagent pads for ordinal scale reporting of relative density, pH, leukocyte esterase, nitrite, protein, glucose, ketones, urobilinogen, bilirubin and hemoglobin/myoglobin.

The intensity of the reaction color of the test pad is detected by measuring the percentage of light reflected from the surface to the test pad. A higher analyte concentration results in greater color intensity, and thus in a lower reflectance value. The reflectance value, expressed as a percentage within a range from 100% (white) to 0% (black), is therefore inversely proportional to the concentration of the analyte in the sample. Specific gravity (refractometry-based) is measured in a flow cell and color is rated with a specific algorithm against the blank pad on the test strip. Data are expressed on an ordinal scale (as ''normal'', ''negative'', ''positive'' or as nominal concentrations) on the reports provided by the instrument, but (quantitative) reflectance data can be exported to a laboratory information system or a network environment.

Calibration of the flow cell and photometer was carried out according to the manufacturer's instructions. The reflectometric assay was calibrated vs. aqueous lithium AcAc (Sigma, St Louis, MO, USA) and acetone standards (Sigma). These calibration curves were used to convert reflectance values (% remission) into concentrations (mmol/L). Linearity was tested in aqueous solutions over the range 0–14 mmol/L (acetone), and 0–20 mmol/L (acetoacetate). Reproducibility was assessed using samples with low and high analyte concentrations analyzed ten times in one run (within-run CV) and on 10 consecutive days (between-run CV).

3-Hydroxybutyrate analysis

For the additional determination of 3HB, the sample was pretreated with 1230 U/L 3HB dehydrogenase (EC 1.1.1.30; Sigma) and 4.64 mmol/L NAD^+ (Sigma) in a glycine-NaOH buffer solution (pH 9.0). 3-Hydroxybutyrate dehydrogenase catalyzes the conversion of 3HB into AcAc, with concomitant reduction of NAD^+ to NADH. This buffer system is compatible with the conditions required for the second reaction step. After 20 min of pre-incubation, the sample is analyzed quantitatively as AcAc on a urine test strip based on the Legal reaction (sodium nitroprusside). The difference between this final reading and the basic reading (without pre-treatment step) is calculated and the result is compared to a 3HB standard (Sigma). The linearity of the method was tested in the same way as the quantitative test strip method in the range 0–10 mmol/L.

Biochemical investigations

3HB in urine and serum was measured colorimetrically according to Williamson (13) using commercial reagents (Sigma) ($n=9$) on a Hitachi 911 analyzer (Hitachi, Tokyo, Japan). Urinary AcAc was assayed according to Williamson (13).

Interferences

Glucose was added in aqueous solution at concentrations ranging from 0 to 110 mmol/L. 3HB was tested in the range 0–15 mmol/L. Electrical conductivity was varied by addition of various NaCl solutions (final conductivity range from 5 to 30 mS/cm).

The effect of urinary pH on the test results was tested by varying the pH of a urine sample containing 0–20 mmol/L AcAc with sodium hydroxide (0.1 mol/L) from pH 5.0 to 9.0 in increments of 0.2 pH units.

Patients

The study protocol was approved by the Ethics Committee of our hospital. A total of 18 diabetic patients (9 males, 9 females, 38 ± 18 years) under insulin infusion pump treatment were enrolled in the study. They were referred to the endocrinology department because of diabetic ketoacidosis. Urine and serum samples were collected for analysis. We studied freshly collected urine samples submitted to our routine laboratory for diagnostic urinalysis. Samples were stored at -20°C upon arrival in the laboratory and analysis was performed within 1 week.

Determinations of renal thresholds for 3HB and AcAc

Renal thresholds for 3HB and AcAc were calculated by comparing serum and urinary AcAc measurements in three female diabetes patients (age 16, 39, and 46 years). All patients had a serum creatinine concentration within the reference range and were under treatment with an insulin infusion pump. The renal threshold was defined as the extrapolated (linear regression) plasma concentration at which the analyte first appeared in the urine.

Statistics

Values of $p<0.05$ were considered significant. The lower limit of detection (14) was calculated as the mean value$-3\times$SD for a blank sample.

Results

Reproducibility

Table 1 summarizes the within- and between-run CVs for high and low concentrations of acetone (1.16 and 14 mmol/L), AcAc (1.3 and 15 mmol/L) and 3HB (1.3

Table 1 Reproducibility of ketone measurements (n = 10) on the URISYS 2400 analyzer on a urine pool spiked with acetoacetate, acetone and 3-hydroxybuyrate.

	Acetoacetate		3-Hydroxybutyrate		Acetone	
	CV, %	Mean concentration, mmol/L	CV, %	Mean concentration, mmol/L	CV, %	Mean concentration, mmol/L
Within-run						
High concentration	11.0	18.7	5.1	9.04	8.2	16.4
Low concentration	3.6	1.13	3.0	1.12	9.2	1.08
Between-run						
High concentration	11.0	16.8	5.6	8.97	10.4	15
Low concentration	5.8	0.93	3.5	1.14	16.1	1.03

High concentration, 15 mmol/L for 3HB and AcAc, and 14 mmol/L for acetone. Low concentration, 1.3 mmol/L for 3HB and AcAc, and 1.16 mmol/L for acetone.

and 15 mmol/L). Within- and between-run CVs for all analytes were between 3% and 16%.

Calibration curves for acetone, AcAc and 3HB

For both acetone and AcAc, close correlation was found between the reflectance readings and the analyte concentration in urine: y (1/ketone field reflectance) = 11.72 acetone (mmol/L) + 5.23 (r = 0.86); and y (1/ketone field reflectance) = 0.51 AcAc (mmol/L) + 2.80 (r = 0.98).

Over a broad concentration range (0–15 mmol/L), 3HB did not show any reaction with the test strips.

For 3HB in urine, the following correlation was found after enzymatic pretreatment: y (1/ketone field reflectance) = 0.18 3HB (mmol/L) + 1.92 (r = 0.95).

Comparison between photometric and reflectance ketone results

Fair agreement was found between the photometric AcAc data and ketone measurements on the URISYS 2400. The following regression equation was obtained: ketone field reflectance (mmol/L) = 0.20 + 0.57 AcAc (mmol/L) (Spearman r = 0.90 and p < 0.0001; Figure 1). A lower limit of detection of 0.13 mmol/L (CV = 3.6%) was calculated for AcAc.

Comparison of the 3HB measurements

Good agreement between the colorimetric 3HB concentration and test strip reading following 3HB dehydrogenase treatment was also observed: ketone field reflectance (mmol/L) = −0.68 + 1.52 3HB (mmol/L) (Spearman r = 0.84 and p < 0.01). Figure 2 depicts the correlation between both measurement procedures.

Interferences in AcAc and 3HB measurement

For AcAc and 3HB determined by quantitative reflectance reading, varying the urinary pH values over a broad range (pH 5.0–9.0) did not result in a significant change in reflectance reading. Glucose concentration did not affect results in the range from 0 to 110 mmol/L. Changing urinary conductivity had no effect on the test strip measurements.

In contrast to colorimetric assays, addition of 3HB up to 15 mmol/L did not affect test results in the reflectometric AcAc test.

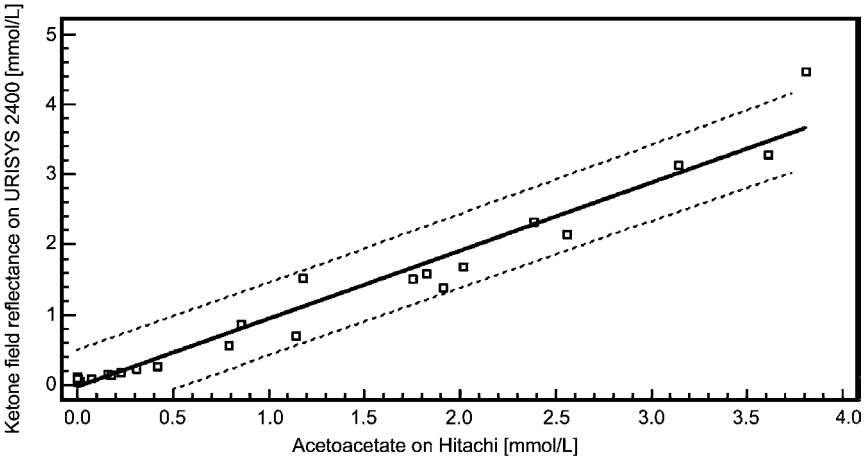

Figure 1 Correlation between acetoacetate results obtained by quantitative test strip analysis and routine chemical determination (colorimetric method) (n = 32): ketone field reflectance (mmol/L) = 0.20 + 0.57 acetoacetate (mmol/L) (Spearman r = 0.90 and p < 0.0001). The two outer lines represent the 95% prediction interval around the regression line.

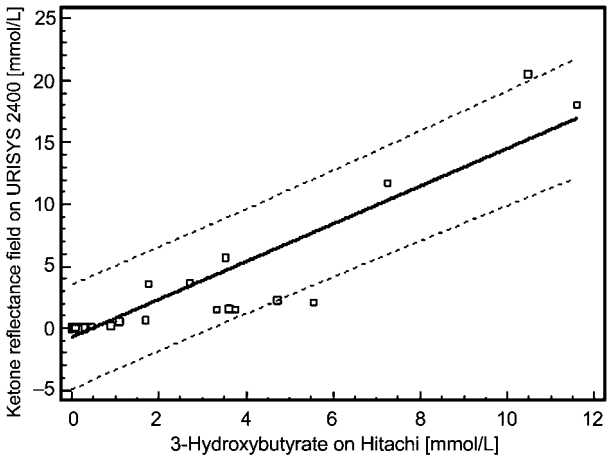

Figure 2 Correlation between 3-hydroxybutyrate results obtained by quantitative test strip analysis and routine chemical determination (colorimetric method) (n = 19): ketone field reflectance (mmol/L) = −0.68 + 1.52 3-hydroxybutyrate (mmol/L) (Spearman r = 0.84 and p < 0.01). The two outer lines represent the 95% prediction interval around the regression line.

Table 2 Acetoacetate and 3-hydroxybutyrate production in diabetics under insulin pump treatment.

Age, years	38 ± 18
Gender, males/females	6/6
Maximal serum AcAc, mmol/L	1.20
Maximal serum 3HB, mmol/L	8.37
Maximal urine AcAc, mmol/L	21.16
Maximal urine 3HB, mmol/L	37.70
Renal threshold for AcAc, μmol/L	38 ± 7
Renal threshold for 3HB, μmol/L	180 ± 70
Urinary creatinine, mmol/L	5.07 ± 3.42

Clinical data

In the diabetes patients, urinary ketone and 3HB excretion was quantitatively monitored under insulin pump treatment. Table 2 summarizes the major biochemical data obtained under insulin pump treatment. For 3HB and AcAc, renal threshold values were 180 ± 70 and 38 ± 7 μmol/L, respectively. Figure 3 depicts the typical evolution of AcAc and 3HB in serum and urine during insulin pump treatment.

Discussion

In this study we demonstrated that the URISYS 2400 automated strip reader is able to provide quick and accurate quantitative measurements of urinary ketones. In the traditional urine test strip, color development is much more intense for AcAc than for acetone, and 3HB is not detected. Thus, in clinical practice, the test path will almost exclusively detect AcAc. However, it should be mentioned that in vivo the highly volatile acetone is mainly eliminated by the lungs (15). Only 1% of acetone is excreted via urine. Moreover, since acetone is a volatile compound, the pre-analytical requirements for obtaining accurate determinations of urinary acetone are very difficult to realize in a routine clinical setting. Isolated increases in urinary acetone concentration have only occasionally been observed following isopropanol intoxication (16).

Additional quantitative determination of urinary 3HB was made possible using a simple pretreatment step followed by reflectance measurement of the ketone test path. Although not indispensable for clinical practice, the latter measurement provides a more complete picture of the patient's metabolic state.

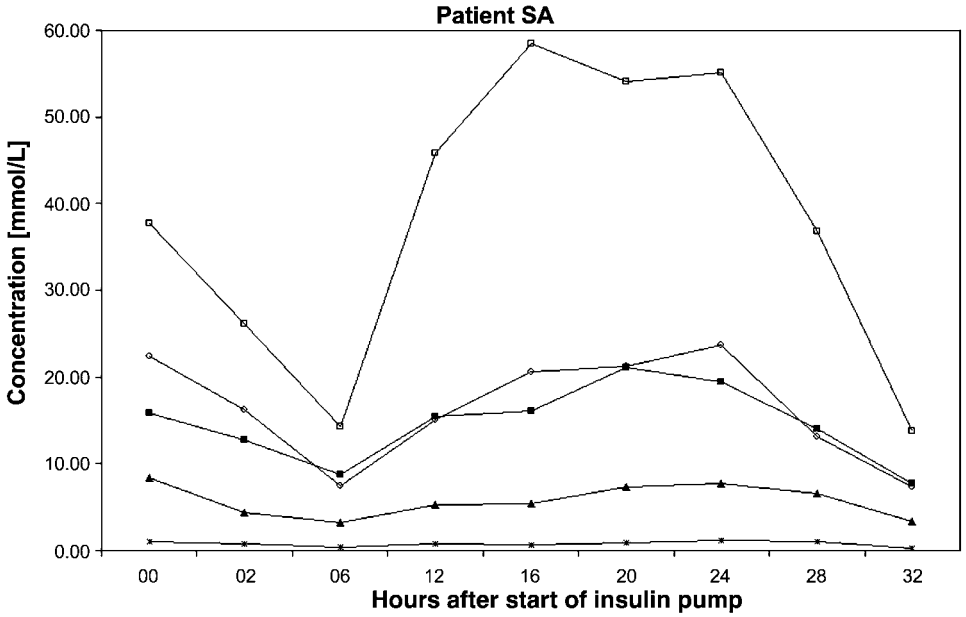

Figure 3 Typical evolution of serum and urinary AcAc and 3HB in a diabetic under insulin pump treatment: □ urinary 3HB (Hitachi); ■ urinary AcAc (Hitachi); ○ urinary AcAc (ketone field reflectance; URISYS 2400); ▲ serum 3HB (Hitachi); * serum AcAc (Hitachi).

When more accurate estimation of ketone production is requested, a conventional ordinal-scaled result might be misleading. Therefore, it is advantageous to have a better estimation of the true concentration of urinary ketones, including 3HB, by means of quantitative reflectance readings. It offers an improved, fast and reliable method that is easy to handle and usable in primary care laboratories and could be of special interest for insulin-dependent diabetes patients.

In the literature, false results have been reported for qualitative ketone measurements using test strips. False positive results are commonly reported when the patient is taking sulfhydryl drugs (e.g., captopril, N-acetylcysteine, mesna, dimercaprol and penicillamine), while false negative results have been reported in the presence of high concentrations of vitamin C.

In the case of diabetic ketoacidosis, test strip results were not significantly affected by variations of urinary pH. Furthermore, no significant effects of sample conductivity and urinary glucose concentration on the ketone test field were noted. In cases of extreme dilute urine, correction of test results (e.g., according to creatinine or conductivity) can be recommended.

In this study, the urinary ketone body concentration agreed well with the colorimetric determination of AcAc. The correlation coefficient for the reflectometric ketone method compared to the routine enzymatic method was 0.90. In contrast to the enzymatic AcAc assay (in which 3HB significantly shifts the reaction equilibrium towards AcAc), interference from 3HB on the ketone test strip was negligible. The latter finding is of importance, since in diabetic ketoacidosis, 3HB is the major ketone body present.

Since urinary ketones represent a mean value of the excretion rate, urine measurements are less sensitive to biological variations than blood measurements. Moreover, due to the very low renal threshold for ketones, urinary ketone concentrations far exceed those observed in blood, which is a major analytical advantage. The stability of ketones in urine is excellent (17).

The detection limit for the URISYS 2400 ketone assay was 0.13 mmol/L (AcAc). The renal thresholds observed for 3HB (180 ± 70 μmol/L) and AcAc (38 ± 7 μmol/L) are well within the reference range of these analytes in serum (30–650 μmol/L for 3HB and 15–220 μmol/L for AcAc) (18). The low renal threshold for both AcAc and 3HB contributes to the good diagnostic sensitivity of their urinary determination.

The American Diabetes Association recommends that anyone with diabetes perform urinary tests for ketones during times of illness or stress, during pregnancy, when blood glucose levels of 17 mmol/L persist, or whenever symptoms of ketoacidosis are observed. The presence of precipitating factors that contribute to the development of DKA would also indicate that ketones should be monitored. While diabetes type 1 patients are at particular risk, ketoacidosis is increasingly recognized as a problem associated with type 2 patients as well, especially those who are insulin-dependent.

In diabetics treated with insulin pumps, checking urine for ketones is important for reducing the misdiagnosis of DKA (e.g., due to undetected leakage of the infusion system, infection, mismanagement of the pump) (19, 20).

In conclusion, quantitative urine test strip analysis provides reliable data on ketone body excretion. The proposed method is simple and cheap, well suited for the routine clinical laboratory and can be used in an emergency setting. The high sensitivity for ketones may allow screening for ketone production in diabetes. In particular, the analysis allows monitoring of the metabolic state of diabetics on insulin pump treatment. Additional sample treatment with 3HB dehydrogenase allows the analysis of 3HB, which yields a more complete picture.

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Chapter 3 C

Alpha 1-microglobulin: clinical laboratory aspects and applications

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Review

Alpha 1-microglobulin: clinical laboratory aspects and applications

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Abstract

Background: Urinary microproteins are becoming increasingly important in clinical diagnostics. They can contribute in the non-invasive early detection of renal abnormalities and the differentiation of various nephrological and urological pathologies. Alpha 1-microglobulin (A1M) is an immunomodulatory protein with a broad spectrum of possible clinical applications and seems a promising marker for evaluation of tubular function. *Method*: We performed a systematic review of the peer-reviewed literature (until end of November 2003) on A1M with emphasis on clinical diagnostic utility and laboratory aspects. *Conclusions*: A1M is a 27-kDa glycoprotein, present in various body fluids, with unknown exact biological function. The protein acts as a mediator of bacterial adhesion to polymer surfaces and is involved in inhibiting renal lithogenesis. Because A1M is not an acute phase protein, is stable in a broad range of physiological conditions and sensitive immunoassays have been developed, its measurement can be used for clinical purposes. Unfortunately, international standardisation is still lacking. Altered plasma/serum levels are usually due to impaired liver or kidney functions but are also observed in clinical conditions such as HIV and mood disorders. 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).

Keywords: Proteinuria; Heavy metal intoxication; Renal disease; Urinary tract infection; Tubular function

1. Introduction

The evaluation of renal tubular function is an important issue in clinical laboratory medicine. Detection of urinary microproteins can help to detect renal abnormalities at an early stage and differentiate the various forms of renal and urological pathology by less invasive techniques. For many years, beta 2-microglobulin has been a golden standard urinary marker protein. However, the diagnostic utility of this protein is hampered by its poor stability at acid pH. The stable microprotein alpha 1-microglobulin (A1M) offers an interesting alternative for evaluating tubular function.

Furthermore, A1M is an intriguing multifunctional immunomodulatory protein. Numerous studies have revealed a broad spectrum of clinical applications. This review aims at giving an overview of recent developments in the basic knowledge, analysis and clinical use of A1M. To accomplish this, we have

Abbreviations: A1M, alpha 1-microglobulin; IgA, immunoglobulin A; IL, interleukin; UT, ulinastatin.

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performed a systematic review in Pubmed of the peer-reviewed literature until November 15, 2003.

2. Structure and function of A1M

2.1. Synthesis and genetics

A1M is encoded by a unique gene encoding both A1M and another protein, bikunin, which has no other known relation to A1M than the co-synthesis [1]. The gene is translated into the A1M-bikunin precursor, which is subsequently cleaved. Bikunin is the small, active subunit of protein/carbohydrate complexes that constitute the inter-alpha-inhibitor family [2].

The human *AMBP* gene has been cloned [3] and has been mapped to the 9q32–33 region [4]. The *AMBP* gene has a weak minimal promoter [5]. A specific enhancer accounts for the pronounced expression of A1M in the liver. In spite of the stable A1M plasma concentration, the expression of its gene is up- or downregulated during inflammation [6,7]. This is in agreement with the presence of cis-acting elements upstream of the gene with potential interleukin (IL)-6-, interferon- and tumor necrosis factor-responsive sequences [5]. The data show a discrepancy between the up- and downregulated *AMBP* gene and the stable A1M concentrations.

2.2. Structure

A1M is a 27-kDa glycoprotein produced by the liver [8]. It was isolated in 1975 [9–11] and has been designated other names, e.g. protein HC [12] and alpha 1-microglycoprotein [13]. It belongs to the lipocalin superfamily of hydrophobic ligand-binding proteins forming an internal ligand-binding pocket [2,14]. A1M is well conserved in evolution and has been found in mammals [1], birds [15], amphibians [16], and fish [17], but not (yet?) in reptiles. Among the lipocalins, complement factor C8-gamma and A1M have the closest relationship [18]. A relationship between epididymal retinoic acid-binding protein and A1M was demonstrated [19].

The protein consists of 183 amino acid residues [1]. A free reactive cysteine side chain at position Cys^{34} is engaged in complex formation with plasma proteins and in binding to a yellow-brown chromophore. The color is caused by chromophore prosthetic groups, covalently bound to amino acid residues at the entrance of the lipocalin pocket [20]. Different chromophores explain the charge and size heterogeneity of A1M [21]. A 282-Da lipophilic substance (a candidate ligand) was co-purified [20]. Several hydrophobic substances, e.g. retinol, were extracted from the protein [22]. A1M is substituted with oligosaccharides in three positions, two sialylated complex type, linked to Asn^{17} and Asn^{96}, and one oligosaccharide linked to Thr^{5} [23]. Asn^{96} has both diantennary and triantennary structures attached in the case of urinary A1M [24].

2.3. The plasma complexes

In human plasma, approximately 50% of A1M forms a 1:1 complex with monomeric immunoglobulin A (IgA) by a reduction-resistant bond between the penultimate cysteine in the alpha-chain and Cys^{34} of A1M [25,26]. Approximately 7% is linked to albumin [12,27] and 1% by a disulfide bond to prothrombin [27]. In rat serum, A1M was found covalently linked to fibronectin [28] and to alpha 1-inhibitor-3, a homologue of human alpha 2-macroglobulin [29]. It is not known where any of the A1M complexes are formed.

Characteristic forms of high-molecular weight A1M are present in pathological plasma: A1M has a tendency to bind to mutated forms of coagulation factors where a free Cys residue has been introduced. Circulating complexes between A1M and factor IX Zutphen [30], factor XII Tenri [31], and protein C mutants [32] have been identified.

2.4. Physiology

After secretion to blood, A1M exists in free form as well as in a variety of high molecular mass complexes. Free, monomeric A1M passes through the glomerulus out into the primary urine, from which it is reabsorbed by the proximal tubules where catabolization occurs [2,33,34]. The endocytic receptor megalin mediates the reabsorption of A1M [34].

Normal urine contains very small amounts of A1M. In conditions with disturbed tubular function, reabsorption of A1M is reduced and increased amounts are found in urine. The plasma concentration

of free A1M is, in contrast to that of A1M-IgA, mainly determined by the glomerular filtration rate (GFR).

With the exception of the central nervous system, the protein is found in most, if not all tissues: liver, plasma, and kidney are major sites of A1M localization [35]. This reflects the major phases of the metabolism of A1M [8]. The protein is present in the perivascular connective tissue of most organs [36,37]. This matrix-A1M is distributed at interfaces between cells and the external environment (blood/tissue, air/tissue, intestinal lumen/villi) as well as at the interface between maternal blood and fetal tissues in placenta. Also in fetuses, a dominant role of liver and kidney in A1M metabolism was found [36].

2.5. Function of A1M

A1M deficiency has not been reported in any species, suggesting that its absence is lethal and indicating its important physiological role [36].

The plasma concentration is markedly constant during inflammation. One possibility is that A1M exerts a constant immunosuppression, protecting resting tissues, but a local immune response may override the suppression of A1M by a cytokine release. Another possibility is that A1M has an increased immunosuppressive role at biological interfaces where it is present at high concentrations. An example of this is the placenta, where A1M (in free form as well as in complexes not found in blood) protects the fetus from attack by the maternal immune system [37]. t-A1M (t = truncated), an A1M species with a free Cys^{34} thiol group, is released from IgA-A1M as well as from free A1M when exposed to the cytosolic side of erythrocyte membranes or to oxyhemoglobin. The processed t-A1M (present in urine) binds heme [38] and participates in heme degradation. A protective role of A1M against the exposed heme on erythrocyte membranes is suggested. A1M is found extracellularly [39,40] and executes its heme-degradation functions outside the cells [38,41]. IgA-A1M serves as a depot of A1M from which A1M is released and processed locally as soon as the erythrocyte ruptures. The size of IgA-A1M prevents the molecule from glomerular filtration and loss from the circulation.

Both free monomeric A1M and high-molecular weight A1M are widely distributed in extravascular compartments [39,40]. A truncated form of A1M which might be identical to t-A1M is associated with the placenta membrane. During inflammation and necrosis, an activation of free A1M and IgA-A1M occurs in ruptured cells. A1M interacts with heme-binding proteins such as cytochrome *c*. The urinary t-A1M may originate from extravascular tissue [42].

A1M plays an immunoprotective/anti-inflammatory role. An inhibitory effect of A1M–collagen complexes on monocyte free radical and IL-1-beta production was described [43], as well as an inhibition of the antigen-induced cell division of peripheral blood lymphocytes [44,45]. Antigen-induced IL-2 production by T helper cell hybridomas [46], leukocyte migration [44], and chemotaxis [47] are inhibited by A1M. A mitogenic effect on resting lymphocytes was observed [45,48,49], which was suppressed by serum [49]. The N-linked oligosaccharides of A1M inhibit antigen-induced lymphocyte proliferation similar to that of intact A1M [50]. A1M binds to the surface of B and T lymphocytes [51], NK cells [51], the histiocytic cell line U937 [52]. The affinity of A1M for its receptor on T cell hybridomas and lymphocytes was low, 10^4–10^5 [48], and 10^7 on the U937 cell line [52].

2.6. Adsorption–adhesion properties

The adsorption of the protein from serum, cerebrospinal fluid, urine and peritoneal dialysis fluid onto polystyrene surfaces was suggested. In urine, the analysis was extended to polyurethane and silicone stents. The ease of desorption of A1M correlated with surface hydrophobicity of the stent biomaterial. A1M was removed from glass by water washing but required a 30% isopropanol wash to desorb from polystyrene, implying attachment via hydrophobic bonding. The adhesion to polystyrene surfaces by *Pseudomonas aeruginosa* B4, an isolate from a urinary tract infection, was strongly associated with the presence of A1M, which may act as a mediator of bacterial adhesion [53,54]. However promising these results, further clinical evidence regarding the role of A1M in foreign body infections, is still lacking.

Similar to the case for bikunin, A1M is a potent inhibitor of calcium oxalate crystallization in a dose-dependent manner. Urinary A1M concentration was significantly lower in calcium oxalate stone formers

than in healthy subjects. The decreased concentration of A1M in calcium oxalate stone formers could be responsible in these patients for an increased risk of crystalluria [55].

3. Clinical laboratory aspects

3.1. Methods

Various immunoassays for the measurement of A1M have been introduced. Early assays for A1M involved electroimmunoassay and single radial immunodiffusion [9,14,56,57]. Later on, solid phase radioimmunoassay followed [58]. Automated immunoassays using polystyrene particles coated with antibodies (allowing "kinetic" assays) as well as enzyme immunoassays have been developed [59]. Analytical sensitivities as low as 0.8 mg/l were reported with a between-run imprecision (CV) of 11–16% [59].

IgA-bound and free forms were assayed using two separate electroimmunoassays [14]. Fernandez-Luna et al. [60] reported a two-step ELISA, whereas Vincent and Revillard [61] used two separate ELISA assays. De Mars et al. [62] developed a simultaneous enzyme-linked immunoenzyme/immunoradiometric assay that involved three different monoclonal antibodies. A competitive ELISA was described for a renal profile involving A1M [63].

Commercially available reagent strips are prepared either with the goal of detecting albumin. Typically, these dipsticks will change color if the protein in urine is at least 400–600 mg/l for microproteins like A1M. Pugia et al. [64] reported on a new total protein dipstick that showed better chemical sensitivity and agreement, especially for low-molecular weight proteins, with a quantitative procedure as compared to other dipsticks.

Jung et al. [65] described an immunochemical test strip for the determination of A1M in urine that could be suitable for screening purposes if methodical improvements were made.

For nephelometric methods, a lowered precision was only described for high urine calcium concentrations (>7.5 mmol/l), probably due to protein denaturation by calcium hydroxide in alkaline urine [60].

Correlation between values obtained with various standards was good. Between-method comparison revealed marked differences possibly because the standards used were of different origins, indicating the necessity of standardisation of the determination of A1M [59] as the case for beta 2-microglobulin.

3.2. Sample stability

In most reports, unlike beta 2-microglobin, A1M was found to be stable in human urine ex vivo at different pHs and therefore suitable as a marker of tubular injury [62,67]. Donaldson et al. [68] found that the stability of A1M, beta 2-microglobulin and retinol binding protein was affected by urinary pH and recommended alkalinisation performed at the time of voiding. This apparent contradiction might be explained by the fact that samples were at room temperature for varying periods in the latter study.

A1M is stable in urine stored with or without preservative at room temperature for 7 days and up to 21 days at 4, −20 and −70 °C [66,69,70]. Stability studies of A1M in native urine stored at −20 °C demonstrated significant decreases of the concentration after 12 months. Addition to urine of a preservative solution containing benzamidium chloride, ethylene-diamene-tetraacetate, tris(hydroxymethyl)-aminomethane and azide preserved A1M [71].

4. Clinical aspects

Table 1 summarizes the most important conditions related to A1M and its concentration. The major clinical application remains the use of urinary A1M as a marker for proximal tubular damage. The other potential applications are less documented.

A1M levels are very stable in several pathological conditions: no significant changes were seen in serum of patients with neoplastic diseases, central nervous system disorders, infections, rheumatoid arthritis and other disorders [56,58,72]. With a few exceptions, altered total A1M concentrations in plasma or serum are always related to impaired liver or kidney functions. A striking exception is the decreased A1M value in an early, non-symptomatic stage of HIV infection [73]. Since the protein is synthesized in the

Table 1
Clinical pathology and A1M

Biological fluid/tissue	Concentration	Condition
Plasma/ serum	Increased	Decreased glomerular filtration rates [75]. In non-IgA myeloma-related renal disease, total and free A1M values for serum correlated well with values for creatinine and beta 2-microglobulin in serum [62]. It can be used for monitoring renal function [77]. In malignant melanoma, the value of the free and IgA-complexed forms correlated with the clinical stage [82].
	Unchanged	Acute phase reactions, neoplastic diseases, central nervous system disorders, infections, rheumatoid arthritis and hepatitis [58,72,119].
	Decreased	Early, non-symptotic HIV infection [73], severe liver insufficiencies [72,74,119], compensated and decompensated liver cirrhosis [58].
Urine	Increased	Impaired tubular function [56,76, 79–81]: heavy metal intoxication. Balkan nephropathy [90]. Following renal colic and chronic ureteral obstruction. Grade of vesicoureteral reflux, detrusor pressure and compliance [99]. Nephropathy in diabetics [92]. Decreased glomerular filtration rates [75].
Mood disorders		A1M and ulinastatin afford a useful objective index when monitoring patients with disorders [105–107].
Liver		Distinction between primary (hepatocellular carcinoma) and secondary liver neoplasms [74].

liver, severe liver insufficiency leads to a decreased serum level [58,72,74]. The plasma concentration of free A1M is, in contrast to that of A1M-IgA, mainly determined by the GFR [75]. Like other microproteins in serum (i.e. cystatin C, beta 2-microglobulin) the plasma concentration of A1M can be used for determination of the GFR even in the creatinine-blind range, due to higher diagnostic sensitivity: a significant positive correlation between serum A1M and creatinine levels was found ($r = 0.75$, $p < 0.001$). Fractional clearance of A1M increases proportionally to the decrease of creatinine clearance [76]. In patients with non-IgA myeloma-related renal disease, total and free A1M values for serum correlated well with serum values for creatinine ($r = 0.816$ and 0.732 for total and free A1M, respectively) and beta 2-microglobulin ($r = 0.791$ and 0.696, respectively) [62] and is one of the parameters that are very useful in monitoring renal function in multiple myeloma patients [77].

4.1. Nephrological disorders

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, A1M, beta 2-microglobulin and Clara cell protein. Since these products are present in the plasma in low concentrations, urine protein excretion in tubular proteinuria is usually < 1.5 g/day. Table 2 summarizes the physical and physiological properties of some proteins commonly used for assessing tubular function.

Free A1M is found in normal urine, whereas none of the complexes can be detected [10,12,78]. The urinary concentration of free A1M is a sensitive indicator of impaired tubular function [76,79–83].

There are many causes of tubular proteinuria. Nephrotoxic substances that damage the tubule include heavy metals (e.g. cadmium and lead) and some

Table 2
Review of commonly used tubular marker proteins

Marker	Molecular mass (kDa)	Endocytic tubular receptor [34]	Remarks
Alpha 1-microglobulin	27	Megalin	Marked stability over a wide pH range no standardisation
Beta 2-microglobulin	11.8	Megalin	Not stable at acid pH no standardisation
Retinol binding protein	21	Megalin	No sensitive assays available (yet)
Clara cell protein	16	Cubilin and megalin	Rarely used
N-acetyl-beta-D-glucosaminidase	>130	?	Lysosomal storage, activity measurement (potential interference)

fluids. Several investigators have measured free A1M and IgA-A1M separately in normal serum or plasma [60–62,71,109].

Reference intervals for A1M are gender-dependent. Moreover, a marked difference was noted in A1M excretion rate during the day. Expressing A1M excretion rate as protein/creatinine is an improved practical estimate of the excretion rates [110,111]. The geometric means (and SD) of protein excretion rates in normal subjects were as follows: 1.11 μg/min (2.22); and A1M, 0.98 μg/min (2.36) [63].

A1M-IgA complex has great range, from undetectable concentrations (urine, colostrum and cervical mucus) up to 59.2 mg/l in blood plasma. Undetectable concentrations of A1M-IgA complex were also shown in serum from patients with IgA immune deficiency and in cerebrospinal fluid from patients with multiple sclerosis. Increased concentration of A1M were noted in bronchoalveolar fluid from a patient with pulmonary alveolar proteinosis, serum from patients with Behçet syndrome and in synovial fluid from patients with gout, chondrocalcinosis and rheumatoid arthritis. On the other hand, the concentrations of A1M-IgA complex were raised only in those patients with pulmonary alveolar proteinosis or rheumatoid arthritis [109]. Takagi et al. [57] found mean A1M concentrations of 20.8 mg/l in synovial fluid, 28.7 mg/l in ascites, 21.5 mg/l in pleural effusion, 2.7 mg/l in amniotic fluid, 8.2 mg/l in cyst fluid and 42.3 ng/ml in cerebrospinal fluid.

6. Post-analytical aspects

Results of measuring urinary marker proteins are still difficult to interpret. Using a parameter set including A1M, it was possible to separate various renal diseases by analysis of second morning urine. In order to compensate for variation due to urinary dilution, usually creatinine excretion is used [62,110,112,113]. A1M was useful to separate primary glomerulopathies from tubulo-interstitial diseases. Application of these techniques can help to detect renal abnormalities at an earlier stage and differentiate the various forms by less invasive techniques [112,113]. A clear separation of primary glomerulopathies from tubulo-interstitial nephropathies was possible at albumin excretion rates between 4 and 1000 mg/g creatinine. All 42 patients with interstitial nephropathy exhibited elevated A1M above 14 mg/g creatinine, whereas 42 out of 45 patients with a primary glomerulopathy and an albumin excretion below 1000 mg/g creatinine showed normal A1M excretion rates. Only at higher albumin excretion (>1000 mg/g creatinine) the A1M concentration tended to be elevated with increasing albuminuria [113].

Various computer-based expert systems for urine protein differentiation have been developed in which A1M data are used for the evaluation of renal tubular function. Based on a data set including A1M, in combination with a test strip screening, the findings of hematuria, leukocyturia and proteinuria can be assigned to prerenal, renal or postrenal causes, using a computer-based expert system for urine protein differentiation (“UPES”) as a decision-supporting tool. In several clinical studies, this reached 98% concordance with clinical diagnoses and was superior to the diagnostic interpretations of experts. UPES has been successfully integrated into the laboratory routine process [114–117]. Similarly, software was developed for classifying patterns of specific urinary proteins. Measurement of urinary albumin and A1M is mandatory where kidney disease is suspected, has to be ruled out, or requires monitoring, even when the total protein concentration is normal [118].

7. Conclusion

A1M is an immunosuppressive protein with an unknown exact biological function. Many unusual and intriguing properties of the protein have been revealed. The protein acts as a mediator of bacterial adhesion to polymer surfaces and might play a role in renal lithogenesis. In the clinical laboratory, sensitive immunoassays allow us to use A1M determination for clinical purposes. Although A1M is not an acute phase protein, changes in the plasma/serum concentrations have been observed in several clinical conditions such as HIV and mood disorders. 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 pa-

drugs (e.g. aminoglycosides, cyclosporine, cytostatic agents and analgesics). Tubular proteinuria is also associated with acute and chronic pyelonephritis, renal vascular diseases, kidney transplant rejection, Fanconi syndrome, and Balkan nephropathy.

Determination of urinary A1M has been used in intoxications with heavy metals: a diagnostic approach to screen for nephrotoxity due to environmental hazards like cadmium should include A1M. Fels et al. [84] demonstrated that a model combining urinary A1M with other glomerular and tubular analytes, correctly classified 71% of workers with a body burden of 15 μg cadmium/g creatinine or more. Additionally, Jung et al. [85] found that in groups exposed to cadmium, either environmentally or occupationally, 22% and 32%, respectively, had urinary A1M values significantly exceeding these from the control group ($p < 0.05$), which was better than all other urinary proteins investigated. A1M was increased in the exposed persons whose cadmium excretion was < 5 μmol/mol creatinine [65]. Combined determination of uncorrected urinary A1M and *N*-acetyl-beta-D-glucosaminidase was recommended for screening to detect cadmium-induced renal dysfunction at an early stage. Certainly in mass screening, this combination performs better than beta 2-microglobulin or retinol binding protein as demonstrated by comparing the correlation coefficients: *r* values for A1M and *N*-acetyl-beta-D-glucosaminidase were significantly greater ($p < 0.01$) than these for the other two proteins ($r = 0.493$ and 0.348, 0.275, respectively) [86].

Also in the case of occupational lead and mercury exposure, urinary A1M was used as an early marker of nephropathy [87,88].

A1M was used as a biomarker for demonstrating damage caused by carcinogens like dinitrotoluene directed towards the renal tubular system. A dose-dependence of the excretion of urinary biomarker proteins with the ranking of exposure was seen [89].

Urinary A1M as a tubular marker and albumin as a glomerular were successfully used as an early marker in the endemic regions of Croatia (Balkan nephropathy) [90]: 90% of diseased subjects had an A1M level of more than 15 mg/g creatinine. This can allow an early diagnosis of tubular proteinuria but extensive clinical and laboratory examinations must be performed for a definite clinical diagnosis.

In idiopathic membranous nephropathy, excretion of IgG and A1M was associated with the extent of tubulointerstitial damage ($p = 0.0087$ and 0.0024, respectively, for score 0 to 1 versus greater than 2). Only A1M excretion was associated with global glomerular sclerosis and arteriolar hyalinosis ($p = 0.0032$ and 0.0004, respectively). Excretion of IgG and A1M has a predictive value for both remission and progression and is useful to identify patients at risk for progression and for whom immunosuppressive therapy is indicated [91].

In addition to albuminuria (which measures glomerular dysfunction), urinary A1M is useful for the early detection of nephropathy in diabetic subjects [92,93]. A1M was used as a marker for the early stages of diabetic nephropathy in NIDDM. The proximal tubules are damaged as early as during the period of subclinical diabetic nephropathy [93].

Increased urinary excretion of A1M may also occur in other diseases without any apparent tubular defect. Among these conditions are severe reductions in the glomerular filtration with accumulation of A1M in blood and a consequent overload of the few remaining nephrons in their capacity to reabsorb proteins.

In renal and bone marrow transplant recipients, serum A1M is a complement to serum creatinine levels in the evaluation of renal function: positive linear correlations were found in both situations ($r = 0.7–0.8$; $p < 0.001$) [94,95]. Urinary and serum A1M are significantly higher in cyclosporine than in azathioprine treatment and can therefore be an early indicator in the choice of the immunosuppression [94,96].

4.2. Urological disorders

Urinary A1M excretion was successfully used in the diagnosis of various urological conditions involving kidney damage. Urinary A1M excretion is diagnostically useful in vesicoureteral reflux patients, increases with higher intravesical–intrapylic pressure, correlates with the decrease in absolute DMSA uptake or with urinary epidermal growth factor excretion and predicts the outcome after the treatment. A1M is also useful in detecting renal tubular damage in patients with outflow disease of the upper tract [97]. In partial ureteral obstruction, urinary

A1M excretion is diagnostic for renal tubular damage, but not for the condition as such [97,98]. Following renal colic and chronic ureteral obstruction, an increase in urinary A1M excretion was observed while creatinine clearance or age had little and gender had no influence on the urinary A1M excretion. A1M excretion was related to the grade of vesicoureteral reflux with a sensitivity of 90%, a specificity of 70% and a predictive value of 97% [99,100].

In the absence of specific symptomatology in children and neurogenic bladder disease patients, early diagnosis of acute pyelonephritis is a challenge. The urinary A1M/creatinine ratio was highly sensitive and specific (98–100% and 100–87% for Ref. [101] and Ref. [102], respectively) and correlated with DMSA scintigraphy. The drop in DMSA uptake correlated ($r=0.758$, $p<0.001$) with the urinary A1M excretion and the excretion was significantly higher in bilateral than in unilateral pyelonephritis ($p<0.02$). The area under the curve of the ROC-curve was significantly higher for A1M than for albumin or gamma-glutamyltransferase (0.895, 0.654 and 0.512, respectively, $p<0.001$). In contrast to cystitis, elevated urinary A1M excretion is seen in acute pylonephritis [100–102].

Mantur et al. [103] demonstrated the usefulness of the urinary A1M/creatinine ratio as a marker of proximal kidney tubule damage in children with *E. coli* and *Proteus* sp. infections.

Unexpected positive results were found in acute prostatitis. Urinary A1M, alpha 2-macroglobulin and albumin provide optimal differentiation between acute pyelonephritis and acute prostatitis (pseudo $R^2=0.83$; log likelihood -30.55, $p<0.000001$) [104].

4.3. Other disorders

The relationship between the urinary contents of A1M and ulinastatin (UT) and the relation between the serum contents of the free forms of A1M and UT in patients with mood disorders is different from that of healthy subjects. The regression plot between scores of the Hamilton Scale for Depression and ratios of the free form content to total content of UT is more informative on the depressive state than that of A1M. Changes in the slope of the regression plot correlating between urinary contents of A1M and UT may afford a useful objective index when monitoring patients with mood disorders [105–107].

Urinary levels of A1M and of UT and the A1M/UT ratio did not differ between controls and patients with Parkinson's disease. The non-existence of a correlation between A1M and UT levels distinguishes Parkinson's disease from other neuropsychiatric diseases such as dementia (Alzheimer-type and vascular dementia), schizophrenia and mood disorder [108].

A1M is a marker for the hepatocytic lineage and therefore could be useful for the distinction between primary (hepatocellular carcinoma) and secondary liver neoplasms [74].

The measurement of A1M and complexes with IgA in plasma has, with few exceptions, not shown any clinically interesting findings. However, in malignant melanoma, the values of the free and IgA-complexed forms correlated with the clinical stage of the tumor [82].

5. Reference values

Determination of A1M in human plasma or serum is complicated by the presence of different forms of the protein. Consequently, reports on normal A1M concentrations in human plasma/serum have varied widely especially since an international accepted standard is not available.

Table 3 summarizes some of the most important existing data for the most widely used biological

Table 3
Reference values for total A1M and IgA-A1M in some biological fluids

Biological fluid	Form of A1M	Reference/cut-off value	Reference
Serum	free A1M	3.5 μmol/l[a]	[62]
	IgA-A1M	2.6 μmol/l[a]	[62]
	total A1M	4.6 μmol/l[a]	[62]
Plasma	free A1M	11.7 mg/l	[109]
	IgA-A1M	59.2 mg/l	[109]
Urine	A1M	0.31 μmol/l[a]	[62]
	A1M	upper 95 reference limit: 1.27 g/mol creatinine (age 18–40) and 2.20 g/mol creatinine (age >40)	[111]
	IgA-A1M	not detectable	[62,109]

[a] Upper limit of observed range.

rameter sets including A1M as marker for tubular damage may help to improve the quality of the test interpretation.

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CHAPTER 4

Flow cytometric analysis

CHAPTER 4: Flow cytometric analysis

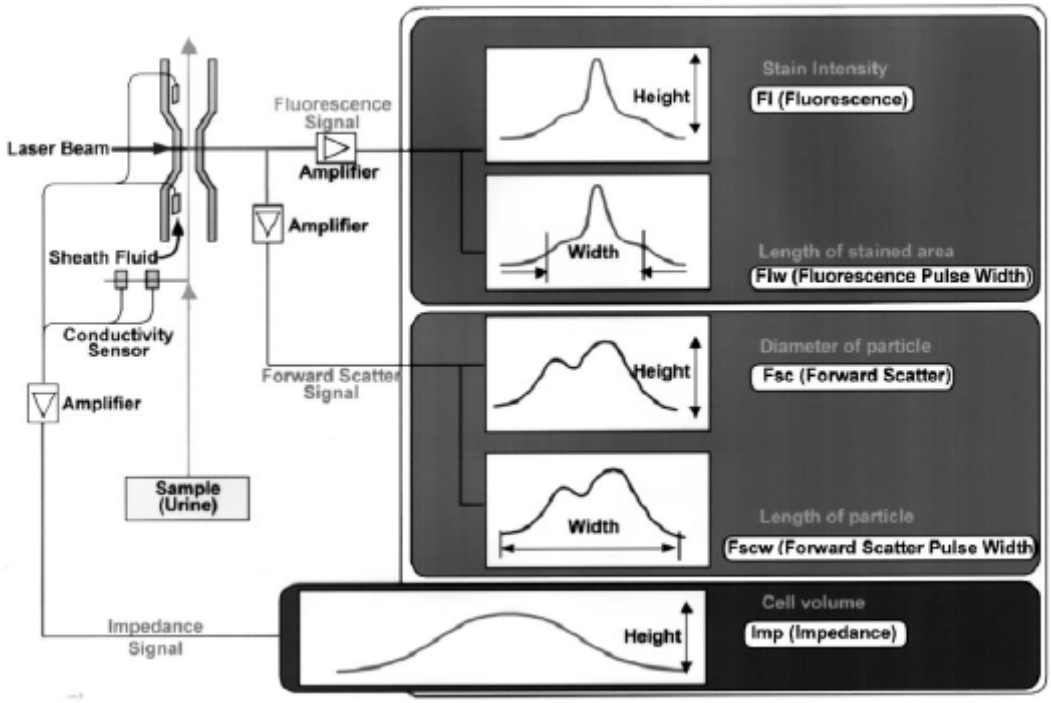
As mentioned in the introduction, flow cytometric analysis of urine has gained wide acceptance in the last years. One instrument dedicated to this analysis is the UF-100 (Sysmex, Kobe, Japan).
Although originally developed for the analysis of urine, the same instrument was evaluated for analysis of other body fluids with an expected low cell count, e.g. cerebrospinal fluid (33), semen (35) and saliva (34).

Instrument description (36)

For identifying urine particles, the UF-100 flow cytometer combines flow cytometry with impedance analysis of urine particles after staining with two fluorescent dyes.
From 9 µL of native urine, several particles are counted and identified: RBC, WBC, squamous epithelial cells (EC), hyaline casts, pathological (inclusional) casts, bacteria, yeast-like cells, spermatozoa, crystals and small round cells (SRC). The latter include mostly renal tubular cells and transitional epithelial cells but also macrophages, intestinal epithelial cells and other occasional cells of the same size.
Results are displayed as scattergrams and histograms and numerical values are reported. Additionally the system provides a flag for RBC size distribution.
It is important to note that the manufacturer of the UF-100 has made modifications to the detection algorithm for bacteria (37). The software used in our laboratory is the older version (version 00-12) in which dual categories exist for bacteria classification as bacteria are detected in two different measurement modes. Small bacteria are mainly detected in a high-sensitivity measuring mode and expressed as ‘‘H-BACT’’ counts while the ‘‘BACT’’ count category refers to larger bacteria or bacterial aggregates which are detected in the normal measuring mode like all other particles. The revised version ads the H-BACT particles to the BACT channel of the instrument hence generating one single result. This revised BACT count is approximately 20 times higher than the original BACT count. The system can thus be more sensitive to noise since thrombocytes and debris may account for false-positive bacterial counts.

In sampler operation mode, urine is automatically mixed, aspirated and diluted. The dilution buffer stabilizes the osmotic pressure of urine within a defined range thus enabling the impedance measurement. Tri-potassium ethylene diamine tetra-acetic acid (K3 -EDTA) chelates amorphous phosphates.

Figure 2: Principle of urine flow cytometry (UF-100)



***This figure is presented in a publication by Delanghe et al* (36).**

Signal generation in the Sysmex UF-100. As the sample passes through the flow cell, a laser beam encounters the particles and specific forward light scatter and fluorescence signals are emitted. The forward scatter intensity (Fsc), reflected in the height of the electronic signal, indicates the diameter of the particle, whereas the width of the forward scatter signal (Fscw) is related to the length of the particle. Likewise the fluorescence intensity indicates the stainability of the particle or a section thereof, and the fluorescence pulse width is related with the length of the stained area. Additionally the impedance signal, illustrating the cell volume, is detected.

The sample is heated to 37°C to dissolve amorphous urates and, after staining with carbocyanine (to stain the cell membrane) and phenanthridine (to stain nucleic acids), enters the flow cell. The particles are hydrodynamically focused; an argon laser beam at wavelength 488 nm encounters these particles and particle-specific forward light scatter and fluorescence signals are emitted. Based on the characteristics of forward scatter (Fsc), forward scatter pulse width (Fscw), fluorescence (Fl), fluorescence pulse width (Flw) and impedance, the urine particles are classified. The UF-100 incorporates an extensive system of manufacturer-defined and user-definable review flags. Samples with particle counts exceeding clinical decision points can be flagged according to user-definable limits. Control on WBC and RBC count is carried out using latex particles supplied by the manufacturer. It is recommended that this material should be used once or twice daily for internal quality control purposes.

Chapter 4 A

Automated flow cytometry analysis of peritoneal dialysis fluid

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Nephrology
Dialysis
Transplantation

Technical Note

Automated flow cytometry analysis of peritoneal dialysis fluid

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Abstract

Background. Recently, the Sysmex UF-100 flow cytometer has been developed to automate urinalysis. We have evaluated this instrument to explore the possibilities of flow cytometry in the analysis of peritoneal dialysis fluid (PD) and have compared the obtained data with those of counting chamber techniques, biochemical analysis and bacterial culture.
Methods. UF-100 data were correlated with microscopy and biochemical data in 135 PD samples. Microbiological analysis was performed in 63 suspected cases of peritonitis.
Results. Good agreement ($P < 0.001$) was obtained between UF-100 and microscopy data for leukocytes ($r = 0.825$). UF-100 bacterial count correlated ($P < 0.001$) with UF-100 leukocyte count ($r = 0.549$). UF-100 bacterial counts were unreliable in samples where interference by blood platelets was observed. Another major problem was the UF-100 'bacterial' background signal in sterile PD samples. Yeast cells were detected by the flow cytometer in spiked samples.
Conclusions. Flow cytometry of PD with the UF-100 offers a rapid and reliable leukocyte count. Sensitivity of the 'bacterial' channel count in predicting positive culture exceeds the sensitivity of conventional Gram stain. Furthermore, additional semi-quantitative information is provided regarding the presence of yeasts.

Keywords: flow cytometry; infection; microorganisms; peritoneal dialysis; UF-100

Introduction

Peritoneal dialysis (PD) is a widely accepted treatment for end-stage renal disease [1,2]. Peritonitis, a frequent and major complication of PD, is associated with high risk of mortality and morbidity [3,4], is one of the most frequent causes of peritoneal catheter loss and discontinuation of PD [5] and leads sometimes to a serious complication like sclerosing peritonitis [6]. Peritonitis-free dialysis remains an important goal for the long-term use of the peritoneum as a dialytic membrane [7]. The diagnosis and effective treatment of peritonitis depends on clinical evaluation and correlation with laboratory examination of the dialysate. Diagnostic criteria of peritonitis in PD patients include any two of the following: cloudy or turbid effluent containing >100 leukocytes/μl, abdominal pain and a positive fluid culture [8,9]. Previous reports have demonstrated problems associated with the diagnosis of peritonitis based solely on these indicators [9]. Various techniques have been used to facilitate the recovery of microorganisms from dialysate, among them the use of selected broth media, processing of large volumes of dialysis effluent by concentration techniques or total volume culture. Nevertheless, microorganisms are not always recovered from dialysate during peritonitis [10,11]. Numerous non-infectious causes of cloudy peritoneal dialysate are known [12]. Fungal causes should be ruled out as early as possible [12]. Other markers have been described [13,14], sometimes not being specific to peritonitis [14].

Microscopy has been the gold standard for counting leukocytes [white blood cells (WBC)] in PD fluid. However, it is imprecise and has wide interobserver variability. Moreover, it is labour-intensive and time consuming. Automation seems the answer to improve both accuracy and productivity of PD fluid analysis.

A flow cytometer-based instrument (UF-100) that performs automated microscopic analysis has been developed. Until now, this instrument has been evaluated for urinalysis [15–17] and analysis of CSF [18] and saliva [19]. Since flow cytometry allows accurate and precise quantitative analysis of cells, we aimed to explore the possibilities of the instrument to analyse PD fluid. In this study, flow cytometric data from PD fluid were not only compared with Fuchs-Rosenthal chamber counting but also with biochemical and microbiological data.

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Subjects and methods

Patients and samples

We studied 135 routinely collected PD fluid samples. Diagnosis of peritonitis could be suspected when symptoms such as cloudy fluid, fever, abdominal pain and rebound tenderness were present. All samples consisted of a collection in a sterile container for routine biochemical analysis and an accompanying dialysis bag for bacteriological analysis. We obtained samples from 13 (35%) male and 24 (65%) female patients with an age distribution of 2–75 years (median: 55 years) admitted to the renal division of the University Hospital, Ghent. Suspected peritonitis was the most important reason of admission. All analyses were performed within 8 h after collection. The dialysis fluids contained NaCl (5.7 g/l), sodium lactate (3.9 g/l), $CaCl_2$ (257 mg/l), $MgCl_2$ (152 mg/l) and glucose (13.6, 22.7 and 38.6 g/l) with an osmolality range of 275–494 mOsm/l.

Sysmex UF-100

The Sysmex UF-100 (TOA Medical Electronics, Kobe, Japan) uses argon laser flow cytometry and measures the sample conductivity. Particles are analysed by electrical impedance for volume, forward light-scatter for size and by fluorescent dyes for DNA (phenanthridine) and membranes (carbocyanine). Pulse intensity and pulse width of the forward scattered light and fluorescence light are measured. From the data, together with the impedance data, the formed particles are categorized by multi-parametric algorithms on the basis of their size, shape, volume and staining characteristics. The results are displayed in scattergrams, histograms and as counts/μl. The UF-100, initially developed for urinalysis, automatically detects and counts erythrocytes [red blood cells (RBC)], WBC, bacteria, yeast cells, crystals, epithelial cells, small round cells, sperm cells and casts. Particles that cannot be classified are counted as 'other cells'.

Biochemical and microscopic investigations

Total PD fluid protein concentration was measured using a pyrogallol red assay (Sopachem, Brussels, Belgium) on a Hitachi 917 analyser (Roche Diagnostics, Mannheim, Germany). Manual microscopic examination of leukocytes was performed in Fuchs-Rosenthal counting chambers. In each sample at least 20 random microscopic fields were examined at 40×10 magnification and the mean WBC cell count was calculated.

Microbiological investigations

Handling of the injection ports and the fluid exchange system was according to standard hospital hygienic rules: injection ports were disinfected with methanol and were allowed to dry for 2 min. Dialysate was then aspirated into a separate sterile container and sent to the laboratory for routine chemical investigation. Microbiological investigations were performed in 63 samples (49%).

The dialysis fluid bags were tested for the presence of bacteria. Before adding brain–heart infusion broth (10 times concentrated) for enrichment, two sterile tubes (50 ml) were sampled from this bag, centrifuged (1000 *g*, 10 min) and the sediment was inoculated to several media: 5% sheep blood agar, chocolate agar, thioglyconate broth (with paraffin), Schaedler agar (anaerobical incubation), Sabouraud agar, Candida ID agar (Biomerieux) and tryptic soy agar with incorporated Tween 80 for disruption of WBCs to obtain a higher bacterial recovery. Identification of isolates was by standard bacteriological methods. The fluid bags and all cultures were incubated and examined daily for 7 days. This way, most organisms are discovered with an easy to perform method [10].

Gram stain was also performed on the sediment. Slides were examined by light microscopy under immersion oil at 100×10 magnification.

Performance and interference studies

To evaluate the linearity in the UF-100 bacterial count channel, we analysed isotonic saline solutions (5 ml) containing one colony from patient isolates of *Escherichia coli* ($n = 3$) and *Streptococcus agalactiae* ($n = 3$). Platelet-rich plasma, obtained after centrifugation of sterile citrated blood for 10 min at 200 *g* ($n = 3$; average platelet count: 485×10^3/μl), was used to study suspected interference of platelets in the bacterial count.

Three physiological saline solutions (5 ml) containing one colony of *Cryptococcus neoformans* were used to evaluate the UF-100 yeast cell count.

Statistics

Data are presented as median and interquartile range (range between 25th and 75th percentile). Agreement between automated cell counts and microscopic data was examined by Spearman rank analysis. Statistical significance was considered at the level of $P < 0.05$. To assess the diagnostic accuracy of WBC, bacteria and total protein, we used ROC-curves and calculated the areas under curves (AUCs) for comparison.

Results

Leukocytes and erythrocytes

The distributions of automated (UF-100) cell counts in negative and positive cultures are summarized in Table 1. Median overall UF-100 WBC and RBC counts were 7 WBC/μl (interquartile range: 4–32 WBC/μl) and 5 RBC/μl (interquartile range: 2–25 RBC/μl), respectively. After logarithmic transformation, good agreement ($P < 0.001$) was found between UF-100 and microscopic counts for WBC ($r = 0.825$) (Figure 1).

Bacteria

Median overall UF-100 count was 31 bacteria/μl (interquartile range: 13–95 bacteria/μl). Similar to UF-100 analysis of cerebrospinal fluid, a 'bacterial' background signal was detected by the instrument in PD fluid samples with negative bacterial culture.

Table 1. Distribution of automated (UF-100) RBC, WBC, bacterial, epithelial cell counts and total protein

Percentile	Culture positive ($n=27$)					Culture negative ($n=33$)				
	RBC (/μl)	WBC (/μl)	Bacteria (/μl)	Epithelial cells (/μl)	Total protein (mg/dl)	RBC (/μl)	WBC (/μl)	Bacteria (/μl)	Epithelial cells (/μl)	Total protein (mg/dl)
10	1	5	18	0	0.00	2	3	11	0	0.00
25	3	9	28	0	0.00	4	4	21	0	0.00
50	9	41	95	1	1.07	7	15	36	1	0.60
75	28	310	178	4	1.93	44	40	69	6	1.24
90	30	1367	290	9	2.67	520	267	229	11	1.81

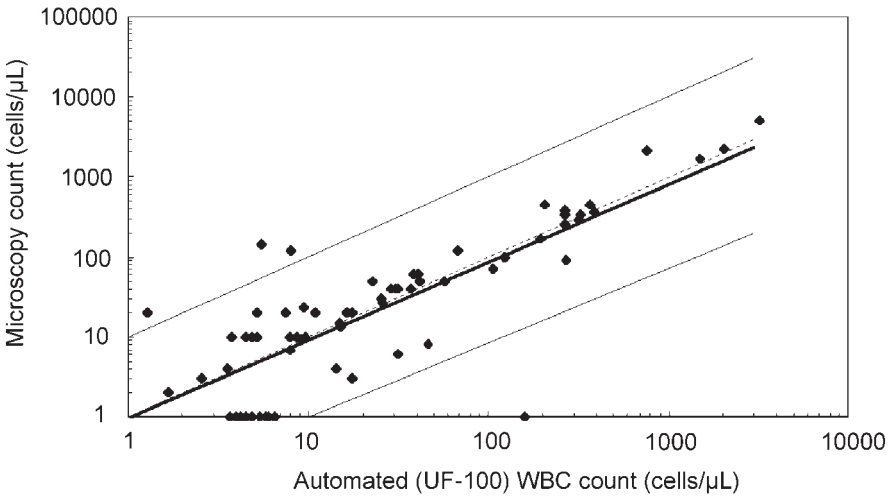

Fig. 1. Correlation (line in full) between flow cytometry (UF-100) and microscopy counts of WBC: log(microscopy WBC; cells/μl) = 0.0067 + 0.97 log(flow cytometry WBC; cells/μl) ($r=0.825$; $P < 0.001$). The dashed line indicates the ideal relationship.

Bacterial cultures were positive in 27 of 63 cultured specimens (43%) and showed coagulase-negative staphylococci ($n=5$), *Staphylococcus aureus* ($n=5$), *Streptococcus viridans* ($n=3$), *Stenotrophomonas maltophilia* ($n=3$), *Corynebacterium* sp. ($n=3$), *Candida albicans* ($n=1$) and mixed infections ($n=7$). Figure 2 represents the automated count in function of the culture result. In contrast to the flow cytometric data, Gram stain followed by microscopy only allowed to detect six of 27 positive cultures (22%).

A moderate correlation was found between the bacterial and WBC counts on the flow cytometer: log(bact; bacterial count/μl) = 1.19 × log(WBC; leukocyte count/μl) + 0.38 ($r=0.549$; $P < 0.001$) (Figure 3).

Epithelial cells

Epithelial cells are also measured by the UF-100. No significant difference was observed in epithelial cell count between culture-positive and culture-negative cases.

Yeast cells

In none of the samples, UF-100 yeast cell counts were above the manufacturer-defined cut-off value (10 cells/μl). However, *C.neoformans* yeast cells were correctly categorized by the UF-100 in three physiological saline solutions to which one colony of *C.neoformans* was added (mean yeast cell count: 28 cells/μl).

Interference studies

Interference studies focused on the UF-100 bacterial count. In 58 of 64 (90.6%) samples with a bacterial count above the 75th percentile, Gram stain and/or culture remained negative.

We assumed possible interference of cell debris in the UF-100 bacterial count. In diluted sterile K_2EDTA blood (1/100 to 1/1000) samples, UF-100 bacterial counts were high (>100 bacteria/μl). Further analysis of platelet-rich plasma ($n=3$) showed that platelets were exclusively categorized as bacteria by the instrument.

ROC-curve analysis

Figure 4 represents a ROC-curve, based on UF-100 analysis, for early prediction of positive PD fluid culture.

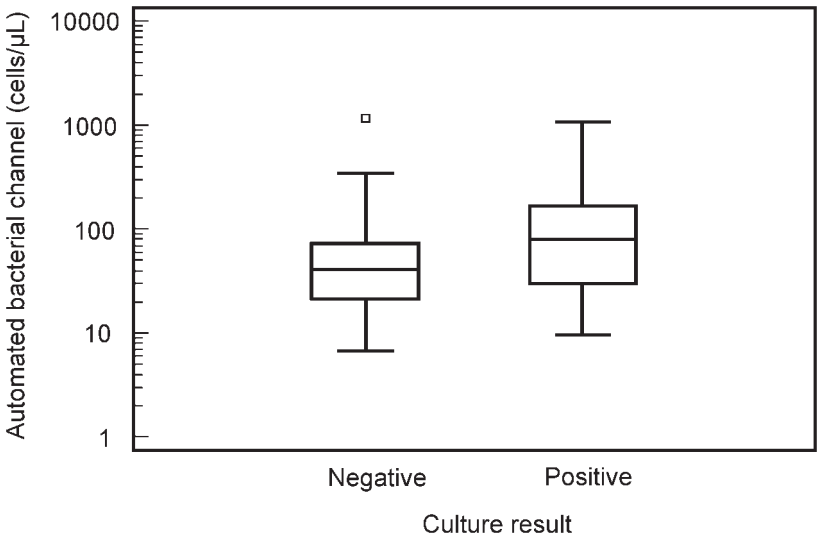

Fig. 2. Box-and-whisker plots of automated UF-100 bacterial count in samples with positive and negative culture results.

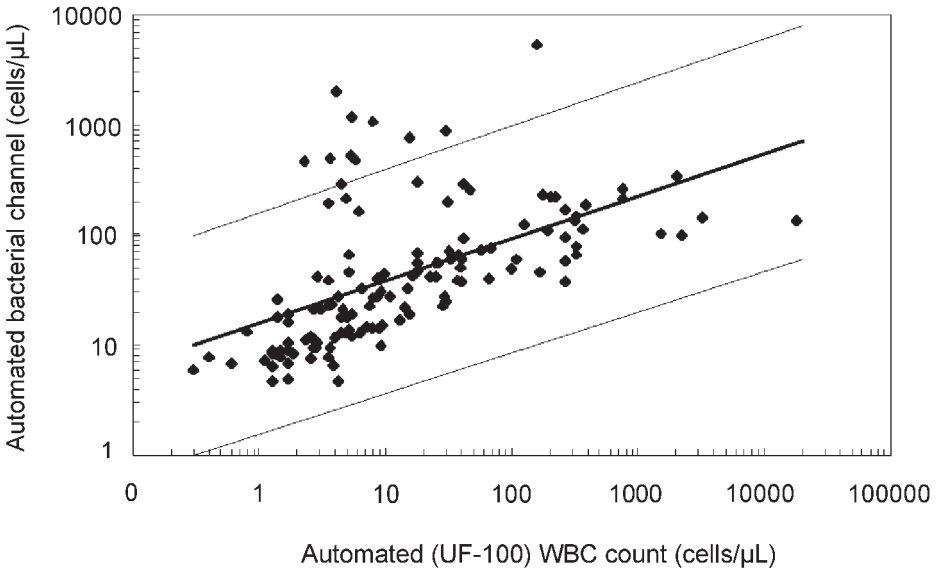

Fig. 3. Correlation between bacterial and WBC counts on the flow cytometer: log(bact; bacterial count/µl) = 1.19 × log(WBC; leukocyte count/µl) + 0.38 ($r = 0.549$; $P < 0.001$).

At a cut-off level of 58 WBC/µl, a sensitivity of 50.0% and specificity of 78.9% was observed. The corresponding AUC was 0.655, which was slightly better than the AUC for bacteria (0.634) and total protein (0.605). Addition of other analytes (bacteria, protein) in the model resulted in slightly improved diagnostic performance with a sensitivity of 75.0%, a specificity of 72.2% and an AUC of 0.743 (Figure 4).

Discussion

We have evaluated the use of a flow cytometer (Sysmex UF-100) in the routine analysis of PD fluid. A good agreement was obtained between WBC counts by the UF-100 and the counting chamber. Comparison with counting chamber techniques, the 'gold standard', is difficult as the latter technique has several steps that may contribute to imprecision and inaccuracy. Especially in the high WBC range, accuracy of microscopic counting can be poor.

As the UF-100 has initially been developed for urinalysis, flow cytometric gating for the detection of leukocytes is focused on the neutrophils, which predominate in peritonitis [10,20]. In most cases and especially when low cell counts are encountered, flow cytometry offers a rapid and reliable WBC count. It has been shown that neutrophils and monocytes are properly classified as leukocytes [18].

Because the UF-100 also reports data on bacteria, it might be tempting to use bacterial counts in reporting probabilities for peritonitis. However, two major

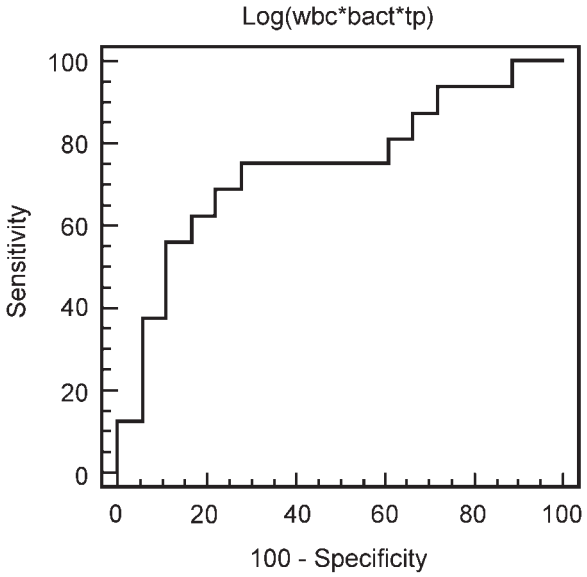

Fig. 4. ROC curve for early prediction of positive PD fluid cultures. Combining UF-100 WBC count, bacterial channel count and total protein measurement results in a sensitivity of 75.0% (47.6–92.6%), a specificity of 72.2% (46.5–90.2%) respecting a criterion of 3.15.

points of concern are involved. First, as was the case in the analysis of cerebrospinal fluid [18], a 'noise' was detected in the bacterial channel, possibly representing cell debris, which cannot be distinguished from bacteria. This is a major concern for flow cytometric analysis of PD fluid, as this body fluid is sterile in normal conditions. Secondly, this parameter has no added value in distinguishing peritonitis because of the wide spreading of the data (Figure 2).

Sensitivity and specificity of the bacterial count in predicting culture were 43 and 77.8%, respectively, which is better than the traditional Gram stain where bacteria were only seen in 22% of positive cultures, a figure which is comparable with previous publications [10,20]. This illustrates the difficulties in culturing PD fluid: the concentration of bacteria is usually low and there is the possibility of pathogens located in the white blood cells [10].

In vitro supplementation of PD fluid with peripheral blood resulted in a small apparent increase in the bacterial channel count despite negative Gram stain and culture. We postulate that interfering particles (probably cell fragments) are measured in the bacterial channel. Cell fragments and bacteria share similar flow cytometric characteristics (low forward scatter, low phenanthridine and carbocyanine fluorescence). Moreover, we demonstrated that blood platelets are exclusively categorized as 'bacteria' by the UF-100.

The additional capacity of the UF-100 to detect yeasts was demonstrated in spiked samples and might help the clinician in the early diagnosis of peritonitis caused by yeasts [12].

In conclusion, flow cytometric analysis is a useful additional tool for PD examination, especially in the emergency setting. It provides rapid (36 s) and accurate data on WBC content of PD fluid. The apparent bacterial count is more sensitive than the conventional Gram stain in predicting positive bacterial cultures and results of bacterial channel count and total protein only have small additional value in the ROC-curve analysis. Absolute flow cytometric bacterial counts should be interpreted with caution since they do not solely represent bacteria. The background 'bacterial' signal in sterile PD fluid is a major point of concern. The possibility to detect yeast cells in spiked samples, suggests that the instrument might help in the diagnosis of fungal peritonitis.

Conflict of interest statement. None declared.

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CHAPTER 5

Combining

techniques

Chapter 5 A

Diagnostic performance of combined specific urinary proteins and urinary flow cytometry in urinary tract pathology

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Original research communication

Diagnostic performance of combined specific urinary proteins and urinary flow cytometry in urinary tract pathology

Running title: Urinary proteins and flow cytometry in urinary infections and hematuria

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Keywords: Proteinuria; Renal disease; Urinary tract infection; Tubular function; alpha 1-microglobulin; alpha 2-macroglobulin.

Non-standard abbreviations: A1M, alpha 1-microglobulin; A2M, alpha 2-macroglobulin; RBC, red blood cell; UTI, urinary tract infection; WBC, white blood cell.

Abstract

Background: Urinalysis comprises three techniques: urinary flow cytometry, test strip analysis and determination of specific urinary proteins. We have investigated the diagnostic possibilities of combining these methods in a cohort of well-documented patients presenting with a broad variety of nephrological and urological conditions.
Methods: Urinary samples of 407 in- and outpatients with nephrological or urological pathology were retrospectively included in our study. Test strip analysis (URISYS 2400), urinary flow cytometry (UF-100) and urinary protein analysis (albumin, total protein, alpha 1-microglobulin (A1M), alpha 2-macroglobulin (A2M)) had been carried out.
Results: In discriminating upper and lower urinary tract infections, A1M and A1M/log(white blood cells) can be used but pathological casts only gives a poor discrimination. The ratio A2M/log(RBC) allows differentiation between cystitis and pyelonephritis while glomerular diseases can be recognised by the log(RBC x urinary total protein). Combining A2M and urinary albumin allows the determination of acute prostatitis.
Conclusions: By using combined parameters provided by various techniques, ratios can be proposed that may be helpful in classifying renal and urological conditions. Possible integration in computer-based knowledge systems may offer valuable information for clinicians.

Introduction

The field of urinalysis has thoroughly been changed by the advent of some new diagnostic techniques. The introduction of urinary flow cytometry (1), (semi) quantitative test strip reading (2) and specific urinary protein analysis (3;4) all have created new diagnostic possibilities. A better analytical precision (5), and a more refined diagnosis (4) have been associated with these new techniques. From a relatively simple basic data set, a number of clinically useful conclusions can be drawn (e.g. selectivity of glomerular membrane defects, differentiation of hematuria, localisation of urinary tract infection). However, the amount of diagnostic information needed for this advanced medical decision making is critical. This has been a major threshold for the introduction of these techniques into routine clinical practice. Recently, a number of attempts have been made to use knowledge-based systems to improve diagnostic decision making (1;4). The majority of the clinical studies involved have been mainly limited to nephrological cases. However, also in urological conditions, the use of specific proteins was shown to be very promising especially in the location of urinary tract infection, the staging of vesico-ureteral reflux and the diagnosis of prostatitis (6;7).

Alpha 1-microglobulin (A1M) is a 27 kDa glycoprotein which is a marker for evaluation of tubular function. Because A1M is not an acute phase protein (its concentration being unaffected by inflammatory reactions) and since it is stable in a broad range of physiological conditions (8), its measurement can be used for clinical purposes. Urinary A1M provides a non-invasive, inexpensive diagnostic alternative for the diagnosis and monitoring of urinary tract disorders (eg. early detection of tubular disorders such as heavy metal intoxications, diabetic nephropathy, urinary outflow disorders and pyelonephritis) (8). Computer-assisted interpretation of parameter sets including A1M as marker for tubular damage may help to improve the quality of the test interpretation.

Alpha 2-macroglobulin is a plasma protein (725 kDa) functioning as a broad-spectrum protease-binding protein (e.g. alpha 2-chymotrypsin, prostate specific antigen). This protein appears in the urine in post-renal hematuria as well as in urinary tract infection (7;9).

Flow cytometry has been introduced for urinalysis (10;11) to obtain quantitative data on urinary particles. The imprecision of urinary flow cytometry is far less than that of conventional urinary microscopy. Combining diagnostic information provided by urinary

flow cytometry and quantitative test strip analysis therefore offers a theoretical basis for the development of diagnostic expert systems (1;12).
In this study we have explored the diagnostic possibilities of combining urinary flow cytometry, test strip reading and the determination of specific urinary proteins (alpha 1-microglobulin and alpha 2-macroglobulin) in a cohort of well-documented patients presenting with a broad variety of nephrological and urological conditions.

Materials and Methods

Patients and samples

A large database was created containing data of freshly collected urine samples submitted to our routine laboratory for diagnostic urinalysis. All samples were received from the nephrology and urology departments involving 637 inpatients and 371 outpatients (n=1008) with an age distribution of 16-83 years (median: 41 years). Samples were completely processed within 2–4 h after arrival. Test strip urinalysis was carried out before flow cytometry analysis (Sysmex UF-100; TOA Medical Electronics, Kobe, Japan) on a URISYS 2400 analyzer (Roche Diagnostics, Mannheim, Germany).
Medical records were retrospectively examined for diagnoses concerning nephrological or urological pathologies resulting in 407 proven diagnoses: glomerulonephritis (n = 17), glomerular diseases (n = 59), pyelonephritis (n = 46), cystitis (n = 257), prostatitis (n = 10), uncomplicated renal calculi (n = 8), uncomplicated/unexplained hematuria (n = 5) and polycystic disease (n = 5). The classification mentioned was made on the basis of expert opinion.
Urinary flow cytometry and test strip reading was carried out on all samples.

Urinary flow cytometry

The Sysmex UF-100 is a urinary flow cytometer-based walkaway instrument that performs automated microscopic analysis. It has been extensively evaluated for urinalysis (1;10;11). The principle is based on argon laser flow cytometry. The UF-100 measures the conductivity and categorizes the particles on the basis of their shape, size, volume, and staining characteristics. The results are displayed in scattergrams, histograms, and as counts per microliter as well as counts per high-power field. The UF-100 automatically detects and counts red blood cells (RBCs), white blood cells (WBCs), bacteria, yeast cells,

crystals, epithelial cells, small round cells (this category includes renal tubular cells and transitional epithelial cells) (5), sperm cells and casts. Particles that cannot be classified in one of the former categories are counted as "other cells".

URISYS 2400

Urine test strip analysis was performed with the automated URISYS 2400. The intensity of the reaction colour of the test pad is detected by measuring the percentage of light, which is reflected from the surface to the test pad. A higher analyte concentration results in a higher colour intensity thus in a lower reflectance value. The reflectance value, expressed as "%" within a range from 100% (white) to 0% (black), is therefore inversely to the concentration of the analyte in the sample. Data are expressed in an ordinal scale (as “normal”, “negative”, “positive” or as nominal concentrations).

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 test strip.

Biochemical investigations

Urinary albumin was measured immunonephelometrically on 389 samples with use of commercially available Behring antibodies on a Behring Nephelometer II analyzer (Dade Behring) standardized against the widely accepted WHO/College of American Pathologists Certified Reference Material 470.

Total protein was performed on all samples by a pyrogallol red method (13) with Standard Reference Material 917a as a standard on a Modular P system (Roche Diagnostics, Mannheim, Germany). Urinary A1M (n=393) was determined on a Roche Modular P System (Roche Diagnostics, Mannheim, Germany). A2M in urine (n=378) was measured immunonephelometrically on a BNII nephelometer (Dade Behring, Marburg, Germany).

Statistics

P values <0.05 were considered significant. Differences between patient groups were evaluated using one-way ANOVA. Diagnostic accuracy was assessed by ROC analysis. All statistical calculations were performed using commercially available statistical software (MedCalc®, Mariakerke, Belgium).

Results

To evaluate preservation conditions of the urine sample tested, the bacteria/WBC ratio can be used for the detection of pre-analytical errors, which is an important issue in urinalysis (12). Stale urine samples are characterized by a high bact/WBC ratio (1).

Since A1M and WBC are disturbed in different pathological situations and their concentration is influenced by different factors, combining the two parameters could be used to differentiate several pathologies. The information contained by both A1M and WBC parameters was expressed as the urinary A1M/log(WBC) ratio. Figure 1 compares the A1M/log(WBC) ratio in urinary tract infections according to their anatomical localisation. Lower and upper urinary tract infections show marked differences in their A1M concentration and A1M/log(WBC) ratio ($P<0.001$).

Figure 2 pictures the ROC curves of several parameters for disciminating upper from lower urinary tract infections. The parameters mostly used are pathological casts and WBC with which we compared A1M and the A1M/log(WBC) ratio. The pathological cast count only gives a poor discrimination between the two anatomical localisations (AUC = 0.522; sensitivity = 69.4%; specificity = 39.3%), whereas A1M showed the highest area under curve (AUC = 0.955; sensitivity = 97.1%; specificity = 92.2%).

Figure 3 depicts the diagnostic possibilities of combining the data of urinary A1M and leukocytes to localise the urinary tract infection. Four quadrants can be defined by drawing two perpendicular cut-off lines at respectively 25 WBC/µL and 10 mg/L for A1M (5) to classify the pathological cases. The lower left quadrant corresponds to non-pathological cases, the upper left is associated with non-infectious tubular pathology (e.g. heavy metal intoxication), the upper right quadrant contains the upper UTI cases and the lower right quadrant the lower UTI patients.

Table 1 represents the discriminative power with matching sensitivity and specificity for several possible parameters in discriminating upper from lower urinary tract infections in cases with significant leucocyturia (WBC>25/µl). These parameters include A1M, WBC, A1M/log(WBC), pathological casts, hyaline casts and small round cells (this category comprises renal tubular cells and transitional epithelial cells) (5).

The urinary A2M values (a biochemical marker for postrenal bleeding) are plotted against the log-transformed urinary RBC count in figure 4 showing the independency of both parameters. The effect representing in this figure as a gap, is due to measurements below the cut-off. Values for alpha 2-macroglobulin above the cut-off are typical for cases of post-renal bleeding, a fact that is inherent to the large molecular weight of the protein. As

expected, no significant correlation can be found. In the patients presenting with significant hematuria (urinary RBC count >25 cells/µL), a further clinical analysis was made according to the anatomical localization of the hematuria. Figure 5 depicts the urinary A2M/log(RBC) ratio in the specific conditions of cystitis, prostatitis and pyelonephritis. A significant difference between two conditions was seen with a median of 0.64 [interquartile range: 0.51 – 1.89] and 1.59 [interquartile range: 0.51 – 3.82] for cystitis and pyelonephritis respectively ($P<0.05$) while prostatitis could not be distinguished from either of the former groups.

Since urinary A2M determinations are not carried out in the majority of clinical laboratories on a routine basis, alternatively total urinary protein was used as a substitute parameter.

To separate glomerular versus non-glomerular disease, the parameter log(RBC x urinary total protein) could be used as shown in figure 6. Glomerular diseases are characterised by hematuria (increased RBC counts) and proteinuria. However, the specificity of both parameters is subject to improvement. Therefore, by combining the two parameters in one formula by using the product, a better discriminatory power can be obtained, increasing the diagnostic power of the variables. In glomerular disease, a significantly higher value is found: 118.49 [interquartile range: 40.90 – 504.58] vs. 32.12 [interquartile range: 11.03 – 111.76] for glomerular and non-glomerular disease respectively ($P<0.05$).

Using the fraction A2M/urinary albumin, acute prostatitis could be differentiated from other pathologies: 0.06 [interquartile range: 0.04 – 0.16] vs. 0.03 [interquartile range: 0.02 – 0.05] for acute prostatitis and other hemoglobinurias respectively ($P<0.05$).

Finally, the effect of the urinary concentration, as expressed by the conductivity and the specific gravity, on the most important analytes was checked. No significant affect was seen on WBC, RBC, A1M, A2M and bacteria.

Discussion

Alpha 1-microglobin is a well-known tubular protein which can be raised in both infectious and non-infectious conditions (8) offering an interesting alternative for evaluating tubular function. It passes through the glomerulus out into the primary urine, from which it is reabsorbed by the proximal tubules where catabolization occurs (14).

On the other hand, leukocyturia, which can be determined in an accurate way (10;11;15), is a good indicator of UTI but unfortunately does not discriminate between upper and lower tract UTI.

In the present study, A1M proved to be a useful discriminator between upper and lower urinary tract infections. This observation is in correlation with a previous report of proximal tubular damage in urinary tract infection in children (16). When using the combined information of A1M and leukocytes as in the form of A1M/log(WBC), no improvement was seen in the sensitivity or specificity, indicating that A1M on itself makes a discrimation possible between upper and lower urinary tract infections in cases of increased leukocyturia. However, by using the combined information provided by A1M and WBC, four quadrants can be defined being non-infectious tubular pathology, upper and lower urinary tract infections and a quadrant containing other urinary tract pathology not involving WBC or A1M excretion. This classification can be helpful in diagnosing tubular and infectious urinary pathology. It must be remarked however that since non-infectious tubular pathology, like heavy metal intoxication, is very rare in our region, no cases could be included in our patient groups so this quadrant represents the part in which theoretically these patients should present.

Traditionally the presence of white blood cell casts on the examination of the urinary sediment has been assumed to be highly specific for and pathognomic of upper urinary tract infection, leading to pursue an aggressive course of therapy (17). However, we found that both casts, reported as hyaline or pathological casts, and small round cells, as measured by the UF-100 flow cytometer, showed a low specificity for localizing urinary tract infection. The poor diagnostic information provided by the flow-cytometrically determined cast counts can be attributed to the low number of casts usually found and the difficult quantification of these urinary structures, not only by flow cytometry (5;15;18). A1M has the advantage of being a stable protein that can be measured on routine clinical chemistry analyzers hence giving rise to low imprecision and high reproducibility (8). This offers advantages over the counting of casts which are more brittle and subjects to greater inter-observer variability.

For the evaluation of significant hematuria (RBC>25 cells/µL), another specific protein (alpha 2-macroglobulin; A2M) as a marker for postrenal bleeding can be used. Since it is not related to or dependent of the urinary RBC-concentration, A2M can provide additional information in hematuria cases. Cystitis and pylonephritis can be distinguished and by combining A2M with urinary albumin concentrations, prostatitis cases can be identified, in

concordance with previous results (7). Since A2M is not a routine parameter in most laboratories, other possible combinations for classifying glomerular disease were investigated. By using log(RBC x urinary total protein), glomerular disease can be separated from other diseases. Although the difference between the two conditions is statistically significant, there still is an overlap zone between the two conditions, as can be seen in figure 6. It should be noted, however, that a log-scale is used to present the data which can give the impression that the medians do not differ a lot while in reality they can differ up to 5 times. Secondly, this still is better then the present situation (e.g. use of casts) giving rise to much worse possibilities in differentiation.

In agreement with earlier observations by Langlois et al, the bacteria/WBC ratio can be used for the detection of pre-analytical errors, which is an important issue in urinalysis (12). Stale urine samples are characterized by a high bact/WBC ratio (1).

Conclusions

Urinalysis has evolved into the modern age of measuring. Flow cytometry has improved the accuracy of the analysis of the urine sediment while 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 (19). Measurement of specific and stable proteins like albumin, A1M and A2M provide additional information about the renal and urological function and structure. When these data can be combined, using a relatively simple data set can provide an extra amount of useful clinical information by integrating results from various new urinalysis techniques (urinary flow cytometry, specific urinary protein analysis, test strip reading).
The proposed ratios of this study can easily be implemented in a Laboratory Information System (LIS). Furthermore, they can be useful for providing additional diagnostic information when implemented in computer knowledge-based expert systems (9;20;21).

Tables

Table 1: Comparison of the discriminative power of several parameters for upper versus lower urinary tract infections for all cases with significant leukocyturia (WBC>25/µL)

parameter	**criterion**	**sensitivity**	**specificity**	**AUC**
A1M/log(WBC)	4.20	94.1	86.3	0.943
A1M	10.6 mg/L	97.1	92.2	0.955
WBC	230 WBC/µL	67.3	50.2	0.574
Hyaline casts	0.1 casts/µL	51,0	67,7	0.543
Pathological casts	0 casts/µL	69.4	39.3	0.522
Small round cells	0.8 cells/µL	81.6	37.3	0.593

Figures

Figure 1: Discrimination between low UTI versus upper UTI and prostatitis

Box-and-whisker plots representing median and 25%/75% percentiles.
Square markers indicate values larger than the upper quartile plus 1.5 times the interquartile range. Round markers indicate values larger than the upper quartile plus 3 times the interquartile range.

* indicates a statistically significant difference ($p<0.05$) with the other patient groups.

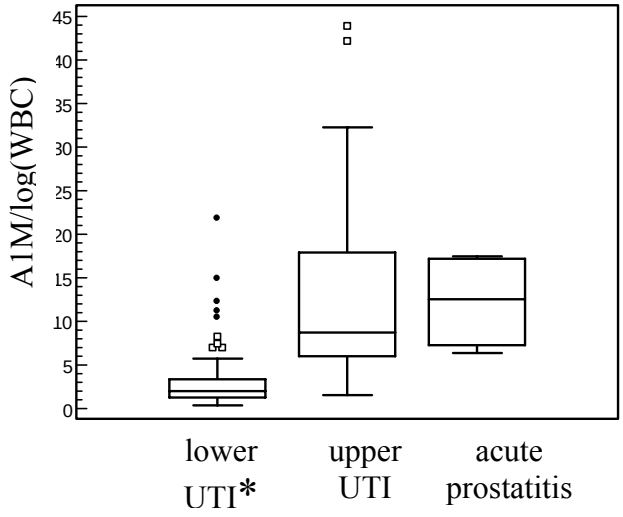


Figure 2: Comparative discriminative power in the localisation of urinary tract infections

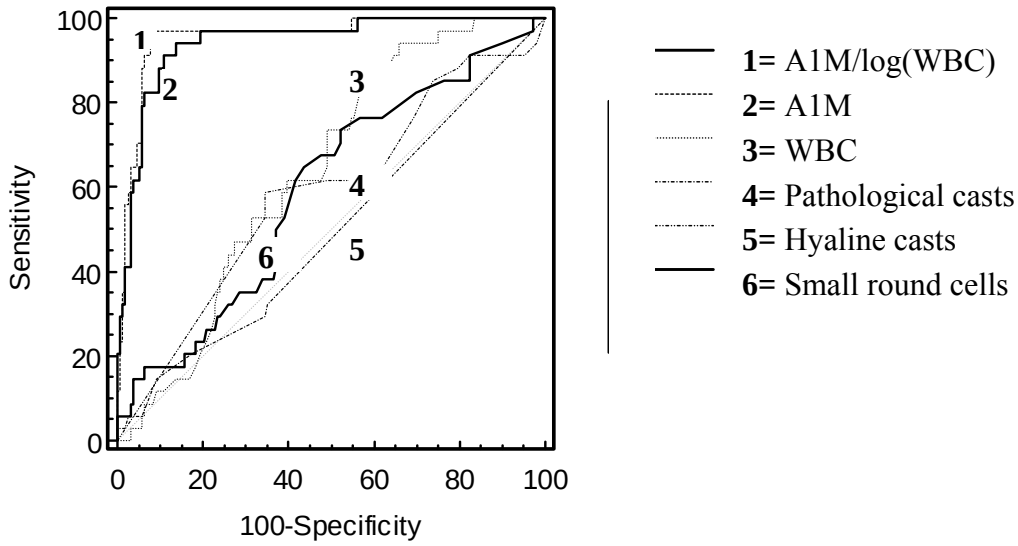
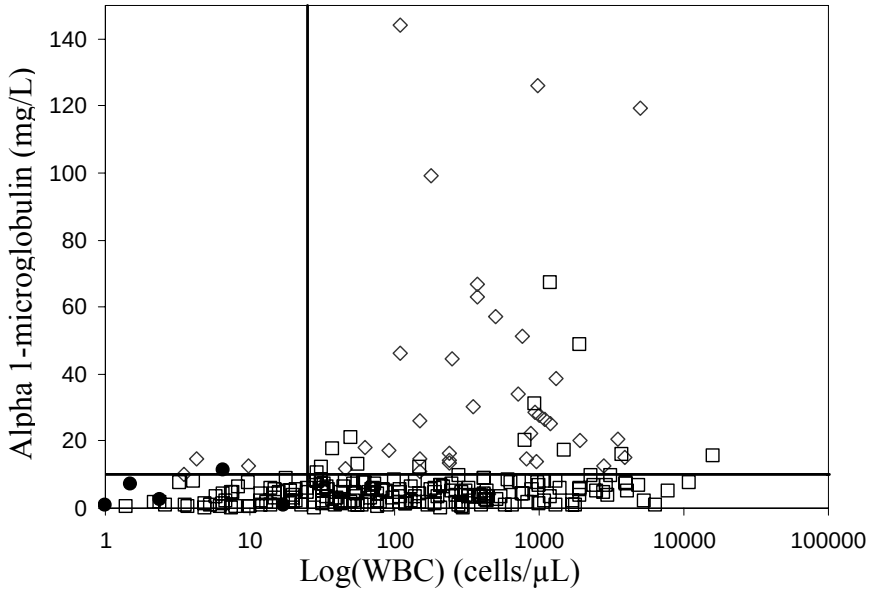


Figure 3: Combining urinary A1M- and leukocyte-data for localising urinary tract infection



●: reflux and uncomplicated hematuria

□: lower urinary tract infection

◊: upper urinary tract infection (pyelo-tubulo-interstitial nephritis)

Figure 4: Urinary A2M versus RBC count in hematuria cases (for RBC counts > 25/µL)

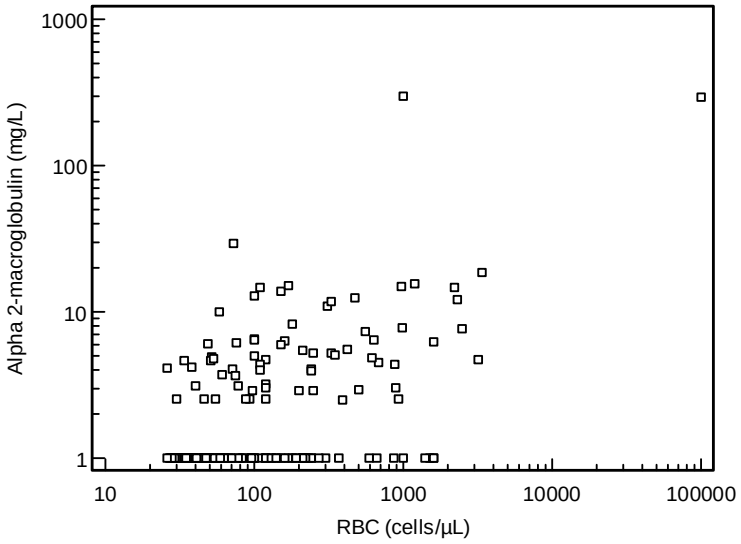


Figure 5: Differentiation of pathology in cases of hematuria (for RBC counts > 25/µL)

Values with a square marker are larger than the upper quartile plus 1.5 times the interquartile range. Values with a round marker are larger than the upper quartile plus 3 times the interquartile range.

* indicates a statistically significant difference ($p<0.05$) between the two marked groups.

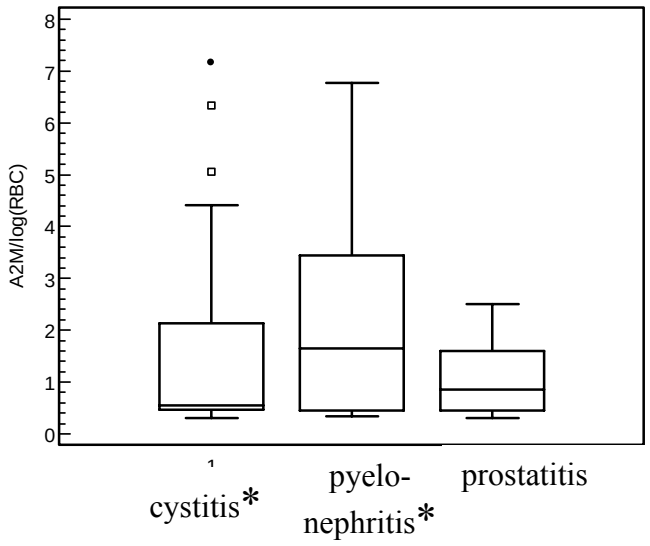
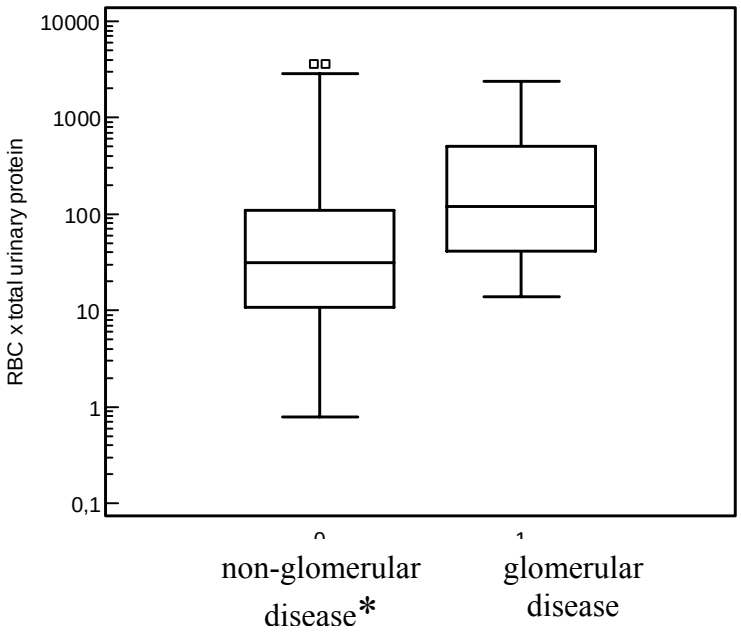


Figure 6: Differentiation of glomerular and non-glomerular pathology

Values with a square marker are larger than the upper quartile plus 1.5 times the interquartile range.

* indicates a statistically significant difference ($p<0.05$) with the other patient group.



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CHAPTER 6

General discussion

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Future perspectives

CHAPTER 6: General discussion & future perspectives

General discussion

Using urine as the sample matrix for analyzing certain analytes does offer some specific peculiarities. It has the advantage of being obtainable in a painless, non-invasive manner; re-sampling is easy and urine is available in sufficient amounts.
However, certain pitfalls and limitations do exist. Urine is not only very easily tampered with by adding additives that disturb measurements or by simply replacing it with a sample from another subject, also the physical properties (pH, specific gravity) may vary significantly resulting in instability of certain parameters. For example, RBC-morphology will be altered in diluted or concentrated urine, RBC and WBC may be destroyed in diluted urine, brittle structures like casts are unstable in alkaline urine, some specific proteins will be degraded in an acid or alkaline urine. Nephrotoxic medications like chemotherapeutics and aminoglycosides will influence the physical properties of the renal glomeruli and tubuli. This contributes to the uncertainty of measurements and the spreading of results. That is the reason why there has been strived for including a large number of patients in the study involved. This should compensate the effect mentioned. Moreover, intra-individual variation is an equally important issue and may exert an effect that is as large as the effect of drugs.
Also prelevation is an important issue especially when bacteriological examinations and (therapeutic) conclusions are to be involved. Therefore specific guidelines for urine sampling have been developed (38).

It is clear, from the literature and from this thesis, that urinalysis has come a long way since the last fifteen to twenty years. The introduction of urinary flow cytometry made urinalysis a more standardised, quicker and less inter-observer dependent technique.
The modern techniques can be used for more than urine analysis alone. The list of body fluids (apart from blood) that have been evaluated on flow cytometry, especially on analyzers developed for urine analysis, now comprises semen (35), saliva (34), cerebrospinal fluid (33) and CAPD (39). Specifically for CAPD, flow cytometric analysis seems to be a useful additional tool for its examination, especially in the emergency setting since it provides rapid and accurate data on WBC content of CAPD fluid. The apparent bacterial count is more sensitive than the conventional Gram stain in predicting positive

bacterial cultures and results of bacterial channel count and total protein only have small additional value in the ROC-curve analysis. Of course, as in almost every technique, there are certain pitfalls so experience in interpreting these techniques is absolutely necessary. As in urine, absolute flow cytometric bacterial counts in PD fluid should be interpreted with caution since they do not solely represent bacteria but may be interfered by cellular debris. This background 'bacterial' signal in sterile PD fluid is a major point of concern. On the other hand, the possibility to detect yeast cells in spiked samples, suggests that the instrument can help in the diagnosis of fungal peritonitis.

The possibilities of the automated urine test strip reading technology have been underused until recently. Not only can it be used in its classical ordinal scaled reading to improve the diagnostic performances of the urine flow cytometer (21), but since the quantitative reflectance reading of a test strip provides reliable data on WBCs, RBCs, glucose and albumin (40) it can also offer the possibility to a more fine-tuned expert system especially to improve the elimination of occasional errors in the WBC and RBC counting channels.

More advanced applications of urine test strips in specific patient groups are emerging. We have shown that in diabetic patients, quantitative urine test strip analysis can provide reliable data on ketone body excretion (41). Quantitative reflectance analysis of the ketone test pad offers a simple and cheap method, well suited for the routine clinical laboratory that can be used in an emergency setting. Test strips show a high sensitivity for ketones allowing screening for ketone production in diabetes. In particular, the analysis allows monitoring of the metabolic state of diabetics on insulin pump treatment. Additional sample treatment with 3HB dehydrogenase allows the analysis of 3HB, which yields a more complete picture.

To increase the diagnostic yield of urinalysis, specific proteins were introduced in the analysis perspectives of this field. A1M seems to become one of the most important analytes in nephrological en urological diagnostics (15-17;19;20;42;43) because of its stability in urine. It is an immunosuppressive protein with an unknown exact biological function. Many unusual and intriguing properties of the protein have been revealed. The protein acts as a mediator of bacterial adhesion to polymer surfaces and may play a role in renal lithogenesis. In the clinical laboratory, sensitive immunoassays allow us to use A1M determination for clinical purposes. 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).

By developing expert systems, the diagnostic information contained in data that are not always easy to interpret will become more accessible also to non-experts. Diagnosis can be suggested and the work-out of the patient's condition could become easier. Such a system, called UPES (29;44), was developed in a nephrological setting. We have integrated results from various urinalysis techniques like flow cytometry, test strip screening and specific urinary protein analysis, and by using a relatively simple data set one can provide an extra amount of useful clinical information. The proposed ratios of our final study can easily be implemented in a Laboratory Information System (LIS) and can be useful for providing additional diagnostic information when implemented in computer knowledge-based expert systems.

Future perspectives

Nowadays, test strips do not play the diagnostic role they could achieve. They are still regarded as an ancient, surpassed technique. However, they are not only cheap and fairly stable, they can also be used in a better way as we showed that their limit of detection for protein is much better than was presumed. Also the relation with flow cytometric RBC and WBC counts and with routine chemical glucose measurements is fairly good. This opens perspectives towards easily accessible screening data: the sensitivity for albumin may allow affordable screening for microalbuminuria, particularly in patients with undiagnosed renal damage. Large-scale global population screening has been the goal of the KDIGO (Kidney Disease Improving Global Outcomes) project of National Kidney Foundation (45). The analytes involved in these guidelines are albumin and creatinine.
Our findings could be important on a large-scale population level in which the increasing incidence of hypertension and diabetes projects increasing renal disease. Early recognition of renal damage by means of screening for microalbuminuria is very important in these patient groups (46-49) and urine test strips may offer a cheap screening although a formal study should be performed to fully explore the possibilities of the albumin test pad in first-line diagnosis.
The aim of the classical multiple test strip is to perform routine chemical analysis in one single operation, yielding maximum diagnostic and prognostic information. Therefore, experts state that a maximum of 10 test areas should not be exceeded and re-evaluated the

usefulness of the existing test pads (50). Indeed, some tests are now obsolete: one could argue about the (diagnostic) usability of bilirubin and urobilinogen on urine test strips. Some experts doubt the usability of the pH test pad although many prefer to keep this to guard the quality of the sample, to identify amorphous sediments and recognise false positive protein tests and cellular lysis. Therefore, some suggest to replace these with test pads that can add diagnostic validity to urine diagnostics. Attempts should be made to expand the use of test strips to more specific and useable analytes like A1M or others that proof to be useful after evaluation (51).

It should also be noted that urine test strips do not allow the detection of Bence-Jones proteinuria. Despite the fact that Bence-Jones proteinuria usually does not present without being associated with proteinuria, this has to be taken into account when judging the results of the protein test pad. This test pad may be an object for future improvements of urine test strips since the only alternatives now are urine electrophoresis and/or immuno-electrophoresis.

Expert systems will become more refined and their use will extend further than the optimization of technical information. On the one hand, one can use them to overcome certain drawbacks of the techniques used (e.g. difficult differentiation between RBC and yeasts by flow cytometry, false positive reading of the hemoglobin pad by myoglobin in test strips, etc.). On the other spectrum, expert systems could develop to become of better assistance in diagnosing urological and nephrological diseases.

Web-based access to expert systems on a central server would certainly contribute to the expansion of knowledge of urinalysis and expertise could be shared with non-experts. One could dream about kidney biopsies becoming redundant but they will probably always be needed for certain indications, for example in detecting immune complexes.

The application of specific proteins in the diagnostic strategies for renal disease will not become a total substitute for kidney biopsy but it will offer an additional tool for gaining diagnostic information in a quick and especially painless way.

Another important gap that still exists is the fact that we need more and better markers for diagnosing cancer of the bladder, kidneys and the prostate. These can possibly be found in urine by PCR based techniques, as shown by recent literature (52).

Another idea could be to look at the surface of cells present in urine and check for certain expression profiles or certain CD-markers. Therefore, a free channel on the standard flow cytometric appliances can be of great help.

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SAMENVATTING

Deze thesis handelt over de beschikbare state-of-the-art geautomatiseerde urine-analyse technieken in een routine laboratorium en bekijkt de mogelijkheden om het gebruik ervan in een klinische en laboratoriumpraktijk uit te breiden en de diagnostische mogelijkheden te verbeteren.

In de huidige praktijk worden resultaten van urine test strips gerapporteerd als semi-kwantitatieve data hoewel de oorspronkelijke aflezing een kwantitatief resultaat is onder de vorm van % reflectantie. We bekeken de prestatie van de testvelden als ze kwantitatief werden gerapporteerd en konden besluiten dat de data van de hemoglobine en leukocytenesterase testvelden complementair waren aan de flow cytometrische resultaten voor deze parameters terwijl de glucose en albumine velden vergelijkbare resultaten gaven met de routine chemische methoden. Dit zou een belangrijk hulpmiddel kunnen zijn in de screening naar microalbuminurie en zou de huidige expertsystemen kunnen aanvullen die nu gebruikt worden om bepaalde tekortkomingen in de flow cytometrie te compenseren.

Met een gewone test strip is het enige keton dat men kan meten acetoacetaat. We bekeken de mogelijkheid om ook de andere ketonen (aceton, 3-hydroxyboterzuur) te meten in urine. We konden, mede door een speciale staalvoorbereiding, alle drie de ketonen meten en vonden een lage nierdrempel voor deze analyten. Dit levert een methode op die een eenvoudige en snelle screening toelaat om ketonen te meten in urine van diabetespatiënten voornamelijk in een spoedgevallensetting. Dit zou kunnen bijdragen tot een completer beeld van de patiënt die naar een diabetisch coma neigt.

Flow cytometrie is een wijdverspreide techniek in de urine analyse en wij konden aantonen dat het ook bruikbaar is als snelle screeningsmethode voor leukocyten in CAPD-vocht. De gevoeligheid van het ‘bacterie’-kanaal in het voorspellen van positieve culturen was beter dan deze van de conventionele Gramkleuring. Ook de aanwezigheid van gisten in het vocht kan onderzocht worden.

Alpha 1-microglobuline bleek, tijdens het opstellen van een review, een van de meest bruikbare parameters in de diagnostiek van renale en urologische aandoeningen. Door het integreren van meetgegevens bekomen door test strip analyse, flow cytometrie en het meten van verschillende eiwitten konden we enkele formules voorstellen die kunnen dienen in de diagnostiek van deze aandoeningen. Door het integreren in expertsystemen zou er aan clinici extra diagnostische informatie geboden kunnen worden.

SUMMARY

This thesis explores the possibilities of the available state-of-the-art automated urinalysis techniques in a routine clinical laboratory setting to expand their use in clinical and laboratory practice and to improve the diagnostic possibilities by combining the information obtained by these individual techniques.

While the original reading data are quantitative reflectance values, urine test strip reading is usually reported as semi-quantitative, ordinal data. The performance and possible advantage of reporting quantitative data was investigated and quantitative hemoglobin and leukocyte esterase reflectance data proved to be complementary with flow cytometric results while glucose and albumin results were complementary to routine chemistry methods. This opens possibilities to use this technique as a screening tool in detecting microalbuminuria and to improve existing expert systems that try to overcome certain pitfalls of urine flow cytometry.

Furthermore, urine test strips can only measure acetoacetate. We explored the possibility of measuring all ketone bodies (acetone, acetoacetate, 3-hydroxybutyrate) in urine with these routine test strips and found that renal threshold levels were low enough to allow quantitative ketone reflectance data to provide a simple and fast analysis, offering affordable screening for the detection of ketone body production in diabetes, especially in emergency settings. This might add valuable information in this setting, to gain a more complete picture of the patient status.

Flow cytometry is also a widely accepted technique for urinalysis and we proved it to offer a rapid and reliable leukocyte count in CAPD-fluid investigation. Sensitivity of the 'bacterial' channel count in predicting positive culture exceeded the sensitivity of conventional Gram stain while additional semi-quantitative information is provided regarding the presence of yeasts.

Specific proteins are used in renal and urological diagnostic strategies and while reviewing the literature about alpha 1-microglobulin, it proved to be one of the most useful analytes in this setting. By integrating information from test strip reading, flow cytometry and proteins, we proposed some ratios that may be helpful in classifying renal and urological conditions. Possible integration in computer-based knowledge systems may offer valuable information for clinicians.

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