DOI: 10.7860/JCDR/2015/8990.5441

Original Article

Biochemistry Section

A Novel Way to Monitor Urine Concentration: Fluorescent Concentration Matrices

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ABSTRACT

Background: The amount of water found in urine is important diagnostic information; nevertheless it is not yet directly determined. Indirectly, the water content in urine is expressed by its density (specific gravity). However, without the diuresis value it is not possible to determine whether the increase in density of urine is due to a decrease in water secretion or an increase in the concentration of secreted substances. This problem can be solved by the use of fluorescent concentration 3D-matrices which characterise urine concentration through the pϕ (or -logϕ) value of the first fluorescence centre.

Materials and Methods: The urine fluorescent concentration 3D-matrix was created by the alignment of the synchronous spectra of the dilution series of urine starting from undiluted (pϕ = 0) to 1000-fold diluted urine (pϕ = 3).

Results: Using the fluorescence concentration 3D-matrix analysis of the urine samples from healthy individuals, a reference range was established for the value pϕ, determining the normal, concentrated or diluted type of urine. The diagnostic potential of this approach was tested on urine samples from two patients with a chronic glomerulonephritis.

Conclusion: The pϕ value of the urine fluorescence concentration 3D-matrix analysis determines whether the urine sample falls within the normal, concentrated or diluted type of urine. This parameter can be directly utilised in sportsmen's hydration state monitoring, as well as in the diagnosis and treatment of serious diseases. An important advantage of this novel diagnostic approach is that a 12/24 h urine collection is not required, which predetermines it for use especially within paediatrics.

Keywords: Diuresis, Fluorophore, Specific gravity, Synchronous spectra

INTRODUCTION

The composition of urine holds information on important metabolic functions of the liver, kidneys and the general state of the internal environment. The main component of urine is water (95%) and the levels depend on the amounts of ingested water and the renal concentration ability of the kidneys [1]. The urine concentration depends on the amount of water released by the kidneys as well as on the levels of secreted inorganic and organic substances. The water content of urine is an important diagnostic marker [2-4], however it is not yet being directly determined for these purposes. From the analytical point of view, no attention is currently being paid to this problem. Indirectly, the water content in urine is determined by the typically correlated values of its density (specific gravity) and osmolarity [5-7]. Generally it is true that the higher the amount of urine, the lower the density and vice versa. Evaluation of density helps to assess the concentration and secretion ability of the kidneys [8]. However it is impossible to determine from density alone, without relevant information on diuresis, whether the increase in density of urine is due to a decrease in water excretion or due to an increase in the concentration of other released substances.

Fluorescent concentration matrices represent a novel analytical approach in the monitoring of urine concentration. Urine is a multi-fluorescent system [9,10] and can be comprehensively characterised through 3D fluorescent analysis. It was first characterised in this way by Leiner [9]. The collection of a urine sample is a non-invasive and easy way to acquire a biological fluid sample, nevertheless complex fluorescent urine analysis is still not a commonly used technique in diagnostic monitoring [11,12]. The most probable explanation for this could be the concentration variability of urine, with there being different concentrations of individual metabolites as well as a nonlinear dependence on the fluorescent intensity over concentration. This problem can be solved by the so called fluorescent concentration 3D-matrices [12,13]. In this paper we present, for the first time, the use of 3D fluorescent analysis of urine for diagnostic monitoring of water content in urine. We demonstrate here that this rapid, simple, cost effective, but powerful analytical approach represents a novel and promising strategy in the diagnosis of serious diseases.

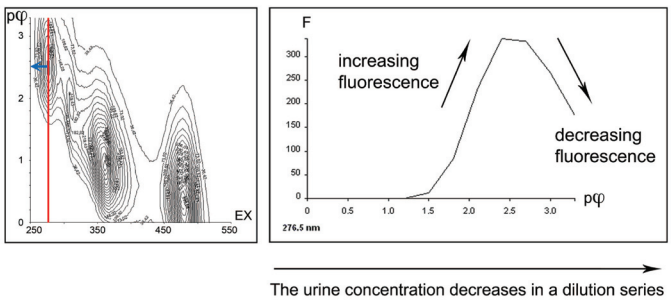
MATERIALS AND METHODS

Urine samples

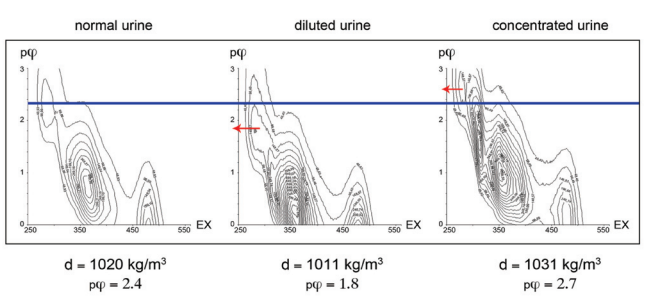
Morning urine samples of patients were routinely analysed in LABMED, a.s. laboratories Kosice, Slovak Republic. Urine samples of adult volunteers were analysed separately. Altogether 110 urine samples were analysed as anonymous waste material, hence no patient consent was needed.

Measurements

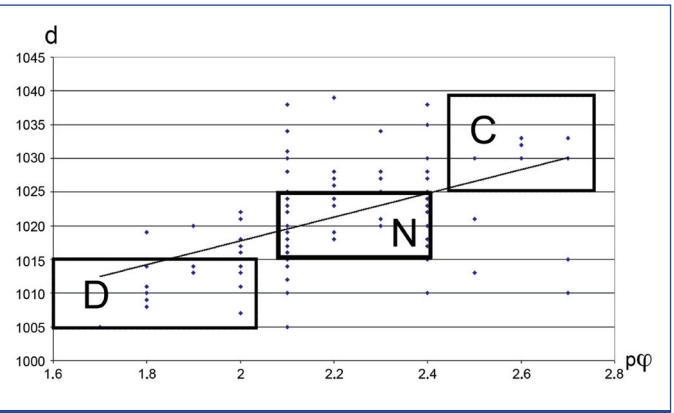
Each urine sample was semi-quantitatively analysed using either the diagnostic strip Heptaphan (Lachema, Czech Republic), testing for the following parameters: pH, protein, glucose, ketones, urobilinogen, bilirubin, blood; or using the diagnostic strip Nonaphan (Lachema, Czech Republic) for the following parameters: pH, protein, glucose, ketones, urobilinogen, bilirubin, blood, density (specific gravity), nitrite. The urine density of patients from the LABMED, a.s. laboratories Košice, Slovak Republic, was determined by a urinometer. The urine density of adult volunteers was determined with the diagnostic strip Nonaphan (Lachema, Czech Republic).



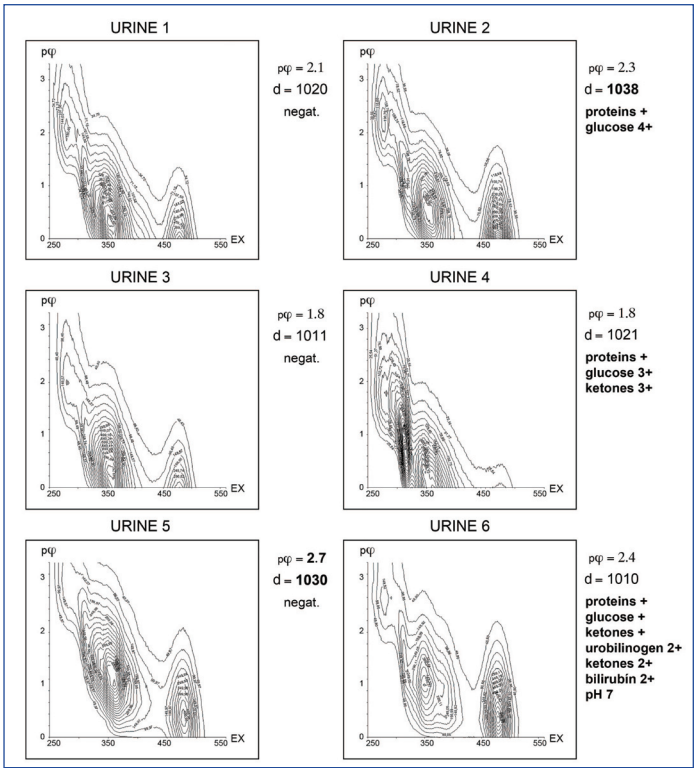
[Table/Fig-1]: Fluorescence concentration matrix (left panel) and a vertical section across the fluorescence centre (right panel; see text for details)



[Table/Fig-2]: Comparison of three fluorescent concentration matrices showing a normal, diluted and concentrated urine sample (see text for details)



[Table/Fig-3]: The relationship between the pϕ value and the density of different urine samples (see text for details)



[Table/Fig-4]: Fluorescent concentration 3D-matrices of different urine samples are combined with their strip analysis results and density (d) values (see text for details)

Before fluorescent measurements urine samples were processed according to Kusnir et al., [12], briefly, 5 mL of fresh urine was centrifuged (10 min, 1100 rpm) and the supernatant was transferred into a new test tube. The set of urine concentrations prepared by water dilution of the supernatant (via geometric progression) represented the following dilutions: undiluted, 1:1, 1:2, 1:4, 1:8.1:16, 1:32, 1:64, 1:128, 1:256, 1:512, 1:1024.

The fluorescent synchronous spectra $\Delta\lambda$=30 nm were scanned using a Luminescence Spectrofluorimeter Perkin Elmer LS 55 (USA). The samples were measured using quartz cuvettes with the path length of 1 cm and the volume of 3.5 ml.

Urine fluorescent concentration matrices

Urine fluorescent concentration matrices were created by software from FLW in lab [12]. The x-y planar projection of all fluorescence values creates a contour 3D-plot (map) – the fluorescent concentration matrix of individual urine samples. The x-axis represents excitation wavelengths (EX) of synchronous fluorescence scanning $\Delta\lambda$ = 30nm and the y-axis represents the negative logarithm of the urine volume fraction (-logϕ = pϕ). Each contour line represents the fluorescence value of 50 arbitrary units, i.e. 5% of the whole fluorescence scale.

RESULTS

A urine sample was first diluted by a geometric progression (see Material and Methods). Individual urine dilutions were expressed by the volume fraction ϕ representing a urine volume/ (urine volume + water volume). For example, the ϕ value of an undiluted urine sample will be one while the ϕ value of a sample diluted 1:1 will be 0.5. Twelve individual synchronous fluorescence spectra of a urine sample, processed by the way described above, were arranged into the 3-D graph based on increasing values of pϕ. In the 3-D graph the x-axis represents the excitation wavelengths of the synchronous excitation spectra (EX), the y-axis urine dilution (pϕ), while the z-axis expresses fluorescence intensity. Urine dilution, mentioned above, is defined by the negative logarithm (–log) of the volume fraction pϕ (analogy to pH). Undiluted urine: ϕ = 1, pϕ = 0; 1000 fold diluted urine: ϕ = 0.001, pϕ = 3. Expression of the concentration in a logarithmic scale enabled a regular arrangement of synchronous spectra with an increment of pϕ = 0.3. Arranged synchronous spectra in a 3-D system produced a planar body which projection into the x-y axis (EX - pϕ) gives rise to a contour map called the concentration matrix of a given urine sample (analogy to excitation-emission matrix). Its contour lines connect places with the same fluorescence.

The contour lines of the fluorescent concentration matrix form enclosed fluorescent centres that differ dependent on the pϕ and EX coordinates. As mentioned above, the coordinate x = EX of the fluorescent centre is a qualitative parameter of fluorophores, while the coordinate y = pϕ depends on the initial urine concentration [Table/Fig-1].

Arrangement of spectra according to increasing dilution (pϕ) gives rise to the appearance of the fluorescence centres in the contour map, a phenomenon reflecting a so called fluorescence paradox: increasing concentration of a fluorophore in the sample increases the fluorescence only to a particular level followed by its decline and disappearance. This contributed to the shape of the concentration matrix with three dominant fluorescent centres in the given coordinate system F = f(pϕ; λEX) [Table/Fig-1], (left panel). A vertical section across the fluorescence centre (EX = 277 nm; left panel of [Table/Fig-1]) illustrates a concentration pattern of the corresponding fluorescence centre [Table/Fig-1], (right panel). In line with the fluorescence paradox, described above, the dependence of fluorescence intensity on concentration is linear only at low fluorophore concentrations (pϕ 3.3 - 2.7 by the urine sample described in [Table/Fig-1]). Undiluted urine (pϕ = 0) in the first fluorescent centre (λEX =277 nm; depicted by the vertical line in the left panel of [Table/Fig-1]) with the highest concentration of metabolites in the urine has no fluorescence, as quenching of fluorescence occurs due to high fluorophore concentration. The gradual dilution of urine tends to increase the intensity of fluorescence, until the maximum is reached at a certain dilution (pϕ= 2.4). This is followed by a decrease in intensity related to the linear dependence of fluorescence intensity on the concentration of the diluted solutions [Table/Fig-1, right panel].

The location of the three dominant fluorescent centres in the coordinate concentration matrix system, mentioned above [Table/Fig-1], depends on the initial concentration of urine. The more

concentrated urine has a higher pϕ value in the fluorescent centre, the diluted urine a lower value. To assess the concentration/water content of the urine sample, we selected the pϕ value of the middle of the first fluorescence centre, marked by the arrow in the left panel of [Table/Fig-1], because it, in comparison to parameters of the other two fluorescent centres, best correlated with the water status of the analysed urines (data not shown). The reference range of the pϕ value marking the middle of the first fluorescence centre was found to be between 2.1 – 2.4. This was indicated by fluorescence spectral measurements of urine in a large number of healthy individuals with a negative strip analysis and a normal density (1015-1025 kg.m^{-3}; data not shown). Urine samples with a pϕ value within the reference range are depicted as normal (N), which reflects a normal amount of water.

Water content in the sample of urine is immediately identifiable by looking at the concentration matrix. Values of pϕ, marking the middle of the first fluorescence centre, higher than 2.4, are typical of concentrated urine, whereas values lower than 2.1 indicate a dilute urine sample [Table/Fig-2]. The numerical values of density (d) taken either from the urinometer or the strip analysis (see Material and Methods) or of pϕ, described below the individual panels, determine the total concentration of a urine sample. The horizontal line represents the normal value of pϕ in healthy individuals. The lower and higher values of pϕ in the diluted and concentrated urine samples, respectively, are indicated by arrows [Table/Fig-2].

[Table/Fig-3] documents the correlation of the density (d) and the value of pφ of the analysed urines. Urine samples with normal (N) concentrations are found within the N boxed region, diluted (D) urine samples are within the D boxed region while the section C (concentrated) represents the region with the highest urine concentrations. The relationship between the pϕ value (x-axis) and the density - d (y-axis) of different urine samples is documented by individual dots.

The combination of the density determination, diagnostic strip analysis of pathological metabolites and the fluorescent concentration matrix analysis gives a good idea of the urine composition. Several examples of such combined analysis of different urine samples are presented in [Table/Fig-4]. The pϕ value, strip analysis results and density (d) values of the six individual urines are indicated on the right of each fluorescent concentration 3D-matrix. The pϕ value gives a good idea of urine density in terms of water content.

Urine 1 represents a normal healthy individual with a negative strip analysis, normal density as well as a “normal/healthy” fluorescence 3D-plot with the pϕ value of 2.1 lying within the reference range (see above). Urines with such parameters are included in the region N (normal) of the [Table/Fig-3].

Urine 2 contains a normal amount of water, based on the pϕ value, but has an increased amount of excreted metabolites – proteins and glucose - reflected in the increase in urine concentration (density). This type of sample is in [Table/Fig-3] located above the N (normal) region.

In urine 3 the low pϕ value of the 3D-plot and the negative urine strip analysis indicate an increased amount of water and a normal (or decreased?) metabolite excretion, respectively. Increased amounts of water and normal levels of metabolites correspond to a decreased urine concentration. An example of this can be identified in [Table/Fig-3] in region D (diluted).

Urine 4 represents a sample with a normal density but curiously with positive proteinuria, glucosuria and ketonuria. Determination of the pϕ value in the 3D-plot of this urine helps explain this anomaly. Namely, the increased water content of this urine (low pϕ) diluted the above mentioned metabolites (positive in strip analysis) and hence the density of this urine is normal. In [Table/Fig-3] the urine is found above region D (diluted).

Urine 5 is a negative urine sample from region C (concentrated; [Table/Fig-3]) with an increased concentration due to its decreased water content (high pϕ).

Finally, urine 6 belongs to the so called anomalous group, sample types of which can be found below the individual selected areas on [Table/Fig-3]. The presence of the given pathological elements in urine 6, determined by the strip analysis (see Material and Methods), as well as its normal pϕ value (the normal water content) on the 3D-plot, should indicate an increased density, but the density value of this urine corresponds to isosthenuria, thus, the fluorescent concentration matrix differing from the expected in this case, could be explained by wrong density measurements of this more alkaline urine (pH 7) by diagnostic strip technology.

In our preliminary study we have also started to test the diagnostic potential of urine fluorescence matrix analysis on urine samples from two patients with a chronic glomerulonephritis over a period of five months. The regular routine urine tests of these patients demonstrated a stable proteinuria between 1.27 to 3.6 g/day combined with a low density (1006-1011 kg/m^3), either due to a substantial reduction in the glomerular filtration rate (GFR), or due to an increase in water excretion. The parallel urine fluorescent concentration matrix analyses supported the latter suggestion by revealing an increased water content (pϕ values between 1.6 – 1.8) as a result of sufficient GFR in both patients examined. According to the National Kidney Foundation classification, in which the level of GFR plays an essential role [14], this finding would diagnose both patients in the early stages of chronic glomerulonephritis, known to be connected with a mild treatment strategy. This preliminary diagnostic conclusion was subsequently confirmed by a number of time-consuming laboratory tests, i.e. blood, urine and imaging, performed for both patients examined in the hospital (K.D., I.L, and A.K. unpublished results).

DISCUSSION

The fluorescent concentration 3D-matrix is a graphical representation of the qualitative and quantitative composition of urine as a complicated fluorescent metabolome containing a number of metabolites present in a range of different concentrations [9,15]. It was created by the alignment of the synchronous spectra $\Delta\lambda$=30nm of the dilution series of urine as described in Results above. More concentrated urine has a higher pϕ value in the first fluorescent centre, diluted urine a lower one [12]. The reference range of pϕ was found to be between 2.1 – 2.4. Thus, depending on the position of the first fluorescence centre within the coordinate system it is possible to identify the pϕ value which determines whether the urine sample falls within the normal, dilute or concentrated type [Tables/Fig-1,2].

The correlation of the density (d) and the pφvalue is documented in [Table/Fig-3]. Urine samples with normal, low and high concentrations are found within the N (normal), D (diluted) and C (concentrated) regions, respectively. Urine samples localised above the latter regions are characteristic of a higher content of secreted metabolites. Since the diagnostic strips only test for a few metabolites, the so-called negative urines may have an increased density due to the increased secretion of an unidentified metabolite. If the metabolites are fluorescent compounds, their presence usually change the shape of the so called “standard/healthy” concentration matrix and enables their identification. On the other hand, in some cases the shape of the concentration matrix significantly differs from the standard one, nevertheless the density remains within the reference range. This discrepancy is due to the extreme sensitivity of the fluorescence as well as the fact that the contribution of different urinary metabolites towards the density and the fluorescence is not always the same [15]. Proteins, for example, contribute to the

density more than electrolytes. On the other hand, urea with almost no fluorescence, is usually present at a concentration of 500 mM (30 g /l) in contrast to 3-hydroxyantranilic acid, fluorescently very significant even at concentrations of 50 μM (7.5 mg/l). Thus, an increase in the excretion of the latter metabolite can change the shape of the concentration matrix but has virtually no effect on urine density.

The urine samples which lie beneath the selected areas [Table/Fig-3], initially look like anomalies. Low water content (high pϕ) together with a low specific gravity can be caused by a decrease in the excretion of some substances or by a wrong density determination by strip analysis. The diagnostic strip works on the basis of ion exchange and acid-base indication [16], hence it does not take non-electrolytes (glucose, urea etc.) into consideration. This may lead to the specific gravity value being lower than it really is. This is also the case with alkaline urines. For example, treating the urine with sodium carbonate shifts its pH value to a basic area and dramatically changes the results of the diagnostic strip analysis of such a urine with respect to density (specific gravity), protein content and some other measured parameters in comparison to the non-treated native urine (our unpublished results).

Thus, from the value of density alone it is not possible to conclude whether the change in urine specific gravity is due to an altered concentration of a certain component or due to the change in water excretion. As described above and in [Table/Fig-4], this problem can easily be solved by determination of the "pϕ value" of a urine sample, which directly identifies the amount of water excreted in urine and might also be of diagnostic significance when it comes to assessing the kidneys of patients with a chronic glomerulonephritis (see Results above). Thus, urine fluorescence matrix analysis is a rapid, simple and cost effective diagnostic test in the monitoring of kidney function and water management in serious diseases.

CONCLUSION

A so called fluorescence paradox, described in the text above, which usually complicates biochemical analysis, was specifically used here to reach an overview of the analysed urine sample by means of the fluorescence concentration matrix. The fluorescence concentration 3D-matrix defines a composition of urine either from a quality (excitation wavelength of the synchronous spectrum – EX characterizing a fluorophore), quantity (intensity of a fluorescence as well as a number of contours) or a concentration (pϕ value) point of view. The value pϕ gives a strong idea of the urine density in terms of water content and can be directly utilised in sportsmen's hydration state monitoring. Moreover, together with density measures or osmolarity it can aid the diagnosis and treatment of serious diseases. An important advantage of this technique is that a 12/24 h urine collection is not required in order to get a better idea of the actual urine concentration. Instead, a small amount of urine is sufficient for the fluorescent concentration matrix analysis, which predetermines this novel diagnostic approach for use with critically ill patients especially in paediatrics.

ACKNOWLEDGEMENT

We thank T. Holland for the reading and correction of this manuscript and J.T. Goncalves for discussions. The work was funded by the European Regional Development OPVaV-2009/2.2/05-SORO (ITMS code:26220220143) and the grant VEGA 1/3364/06.

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FINANCIAL OR OTHER COMPETING INTERESTS: As declared above.

Date of Submission: **Feb 18, 2014**
Date of Peer Review: **Oct 20, 2014**
Date of Acceptance: **Nov 13, 2014**
Date of Publishing: **Jan 01, 2015**