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The potential of autofluorescence spectroscopy to detect human urinary tract infection

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ABSTRACT

Urinary tract infections (UTIs) are known to alter the normal urine composition which, in principle, can lead to changes in urine autofluorescence. This paper describes the study of human urine (normal and UTI) by using UV fluorescence excitation/emission matrices and synchronous spectra and proposes a method of diagnosing UTI without any sample preparation. The method is based on excitation in the shorter UV region (250–350 nm) which shows good discrimination between the normal urine and UTI samples. The synchronous scans with an offset of $\Delta\lambda = 90$ nm were also able to differentiate between normal urines and UTI samples. These differences were observed even though the two known major urine fluorophores, tryptophan and indoxyl sulfate were present in the normal urine and UTI samples in similar concentration as established by HPLC analysis. Although the identity of substances responsible for the altered autofluorescence in UTI is not established, our study shows that autofluorescence has the potential to differentiate between normal human urine samples and those with UTI.

1. Introduction

Human urinary tract infection (UTI) is one of the most common infections in humans [1]. It is estimated that nearly 150 million instances of UTI occur per year globally, causing more than 6 billion dollars in direct health care expenditure [2,3]. Many microorganisms are known to cause UTI. Among the common bacterial pathogens associated with UTI are the Gram negative bacteria *Escherichia coli*, followed by Proteus and Klebsiella and Gram positive Staphylococci and Enterococci [4].

Several tools have been developed for diagnosing UTIs. Near patient tests in primary care most widely utilize dipsticks [5]. In a clinical laboratory setting, tests for UTIs include urine sediment analysis and quantitative bacterial culture [6–8]. In recent years, several novel approaches have been attempted for diagnosing UTI, which include real time PCR, biosensors, immuno-chromatography strips and capillary electrophoresis [9–11]. Although pathological and physiological changes are known to alter the autofluorescence of urine, there is a shortage of studies investigating autofluorescence as a diagnostic tool for UTIs [9–13]. Three key earlier publications in this field [9,10,14] are discussed below.

Leiner et al. analysed various dilutions of urine in the ultraviolet region and used fluorescence excitation–emission matrices yielding the complete fluorescence characteristics in a 3D form [14]. They reported that the fluorophores contributing to the blue-green region of the fluorescence spectrum of urine are tryptophan and its metabolites. They also reported the overall topography of the 3D urine fluorescence spectra and proposed that pattern recognition is a useful method for screening urine. Kusnir and Leskova introduced concentration–dependent excitation–emission matrices for analysing autofluorescence of human urine [9]. They showed a difference in the spectra of human urine from normal individuals and those with hepatopathy and autoimmune thrombocytopenic purpura. However, they did not study the spectra of bacteriuria samples.

Dubayova et al. were the first to report the differences between the synchronous fluorescence spectra (SFS) of diluted urines from normal people and those with UTI or renal diseases [10]. They suggested that the observed spectral variation could be due to the presence of proteins. Proteinuria is common in many renal conditions including UTIs, however, it is not a consistent feature in all UTIs [15]. Thus, testing of diluted urine samples with the focus on protein, by choosing a single offset value of $\Delta\lambda = 30$ nm for SFS recording, as done by Dubayova et al. could result in misleading diagnostic information. Additionally, UTI are also associated with other physico-chemical changes in the urine such as changed pH and a variation in the concentration of indole and other fluorescent compounds, for example, xanthine and neopterin [16,17]. Fluo-

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rophores like tryptophan, neopterin, indoxyl sulfate and xanthine generally have large Stokes shift [14]. Thus an offset of $\Delta\lambda = 30$ nm in synchronous scans is not sufficient to capture effectively the spectral features of these variations.

We report here the utilization of excitation/emission matrices (EEMs) to capture the three-dimensional details of the spectral features of undiluted urine samples from healthy individuals and those with UTI. The measurement of fluorescence was chosen due to its high sensitivity in detecting variations in the concentration of substances [18] and because of its simplicity and case of adaptability for automation and high throughput sampling. We emphasize that earlier studies have focused mostly on analysing diluted urine [9,10,14], to avoid the concentration quenching effect due to the high concentration of fluorophores in urine [9]. However, dilution of urine can result in loss of information related to the fluorophores which are present in low quantities. In our study, spectra of undiluted urines were analysed because of two reasons: (a) to develop a simpler testing protocol that eliminates the dilution step and (b) to be able to analyse autofluorescence contributed by fluorophores which are present in low concentrations (e.g. biopterin, neopterin, folic acid).

We also report on the quantification of two key indole compounds (indoxyl sulfate and tryptophan) by using HPLC, in an attempt to explain their role in urine autofluorescence. The aim of this paper is to assess the potential of urine autofluorescence to be developed as a simple and rapid diagnostic tool for UTI.

2. Experimental methodology

2.1. Urine samples

Urine samples were collected from a large pathology laboratory where they were analysed for pH, protein, glucose, bilirubin, nitrate, specific gravity, blood, ketones, urobilinogen and leukocyte esterase. The presence of red blood cells, white blood cells, casts, epithelial cells and crystals was also tested for these samples (by using iQ200 Sprint, IRIS Diagnostics Division, Chatsworth, CA). Semi-quantitative culture on blood agar and chromogenic agar plates was also carried out to test for the presence of bacteria.

A total of 154 samples were selected for the study. They were chosen in such a way that 87 urine samples which showed no abnormal laboratory findings (*i.e.* they were culture negative as well as negative for other abnormal findings), served as a control group of normal samples. The remaining 67 samples with $\geq 10^5$ colony forming units of *E. coli* per ml, served as bacteriuria samples. All the urine samples were stored in the refrigerator (at 4 °C) and examined within 48 h after collection. The urine was diluted by using double distilled water, as required.

2.2. Measurement of autofluorescence

2.2.1. Excitation/emission matrices

Whole uncentrifuged urine (3 ml) was used for measuring autofluorescence using a Fluorolog Tau3 system (JY Horiba, Edison, NJ) in 10 mm quartz cuvettes at room temperature. The fluorescence emission spectra for excitation at various wavelengths, *i.e.* excitation/emission matrices (EEMs), were obtained in the following ranges, 250–450 nm for excitation (Ex) and 310–750 nm for emission (Em). These three-dimensional plots of fluorescence intensity as a function of excitation and emission wavelengths represent the complete fluorescence characteristics of the sample. In order to cover the broad spectral region of relevance in the most time-efficient way, separate smaller EEMs focused on regions of particular interest were obtained and digitally integrated into a single EEM using MATLAB software.

2.2.2. Synchronous fluorescence spectra

SFS measurements were taken for undiluted and diluted (1:30) urine samples using Fluorolog Tau3 system (JY Horiba, Edison, NJ). SFS of urine samples were analysed using two different offset wavelengths of $\Delta\lambda = 90$ nm and $\Delta\lambda = 30$ nm and cuvettes with two different path lengths, *i.e.* 10 mm and 4 mm

In EEM and SFS measurements, the spectral band passes were 2 nm in both excitation and emission. The spectra were corrected for optical system response.

2.3. HPLC analysis of the concentration of indoxyl sulfate and tryptophan in normal and bacteriuria samples

Urine samples were prepared as per a previously reported method [19]. Stock solutions (1 mg/mL) of tryptophan and indoxyl sulfate standards were prepared in the mobile phase.

A Shimadzu HPLC system consisting of a LC-10 AVP pump with a SPD M10AVP photodiode array detector and an autosampler was used. Chromatographic separations were performed at ambient temperature (23–25 °C) on a Waters 4.6 × 150 mm (particle size – 3.5 μm), SunFire C18 column connected with a guard column. The mobile phase consisted of 0.04 M acetate buffer at pH 4.5 with 5% acetonitrile. The total run time was 60 min at a flow rate of 0.8 ml/min [19]. Indoxyl sulfate and tryptophan were detected by using absorption at 280 nm.

3. Results and discussion

3.1. Excitation/emission matrices (EEMs) of undiluted normal human urine and undiluted bacteriuria samples

EEMs are an accurate way to compare these two groups because they cover the broad spectral region of relevance. In the EEMs of undiluted human urine, it is practically impossible to attribute the fluorescence (emission) peak to a specific fluorophore because many fluorophores in urine possess similar spectral characteristics, and there are intervening effects that affect the spectra such as energy transfer, concentration quenching, inner filter effect, etc. [9]. Therefore the separation of the composite spectrum into the components corresponding to single fluorophores has not been attempted, and our discussion focuses on the overall features of the mixture of all fluorophores present in urine. A comparison of the excitation/emission matrices of 13 samples of undiluted normal human urine and 13 bacteriuria samples clearly showed that undiluted normal urine samples had an emission peak around 440 ± 15 nm when excited at 355 ± 15 nm, whereas undiluted bacteriuria samples had a dominant emission peak around 425 ± 10 nm when excited at much shorter wavelength, *i.e.* at 330 ± 20 nm excitation (Fig. 1a). One example each of excitation/emission matrices of undiluted normal urine sample and undiluted bacteriuria sample with different spectral features is shown in Fig. 1b and c respectively.

In addition, the bacteriuria samples showed a weak fluorescence emission peak emerging at 380 ± 15 nm with 280 ± 10 nm excitation which was not observed for the normal samples (Fig. 1a and b).

3.2. Comparison of synchronous fluorescence spectra (SFS) of undiluted normal human urines with bacteriuria samples using a $\Delta\lambda$ = 90 nm wavelength offset

The SFS technique provides limited information as compared to EEMs (located along an intersection of a 3D EEM plot with a single vertical plane). However, SFS scans are faster than EEMs, and they can be effective for obtaining reasonably well separable data for several compounds in a mixture with a single scan [20].

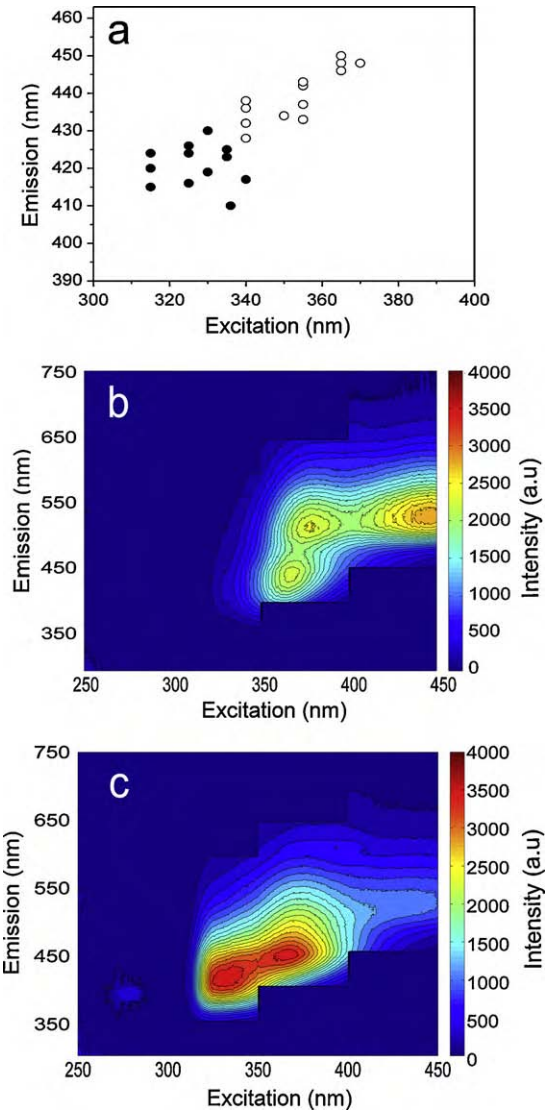

Fig. 1. (a) Localisation of highest excitation/emission peak of 13 undiluted normal urine (○) and undiluted bacteriuria samples (●); (b) example EEM of an undiluted normal human urine sample and (c) example EEM of undiluted bacteriuria sample. *Note*: The region between 250 nm and 300 nm excitations is not visible in the matrix due to intensity scale being adjusted to show the main feature.

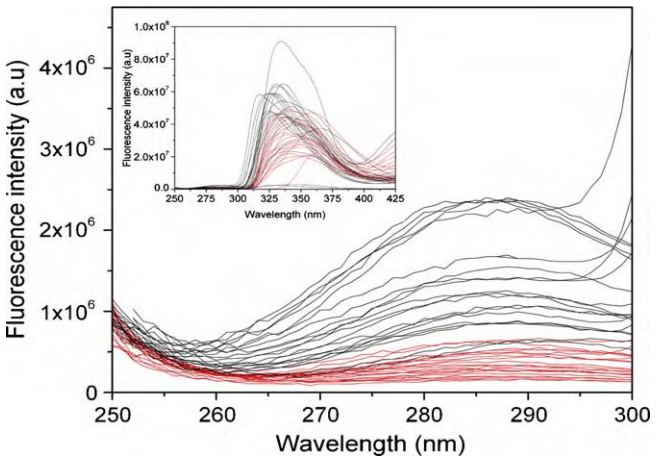

Fig. 2. Comparison of SFS ($\Delta\lambda = 90$ nm) of undiluted normal human urines (–) with undiluted bacteriuria samples (–) measured in 10 mm cuvette for 250–300 nm excitation. The inset shows the SFS ($\Delta\lambda = 90$ nm) of the same samples for 250–425 nm excitation. Each curve represents a different urine sample.

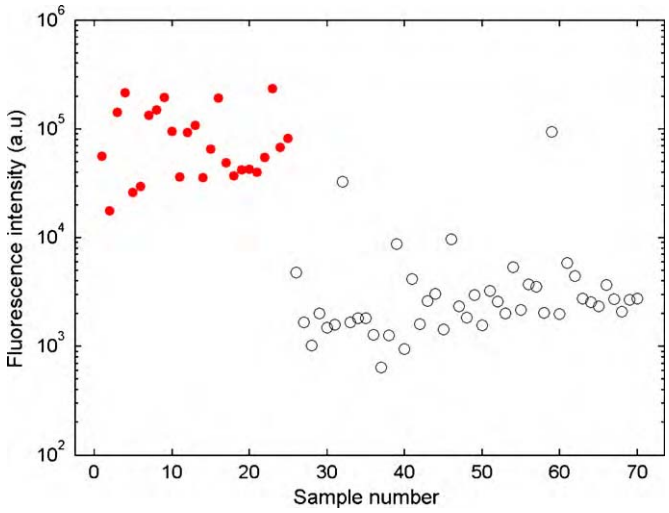

Fig. 3. Comparison of maximum fluorescence intensity (emission intensity) for 45 undiluted normal human urines (○) and 25 undiluted bacteriuria samples (●) at 290 nm excitation wavelength. $P = 2 \times 10^{-12}$, by Kolmogrov–Smirnov (KS) test.

As the two spectral features that differentiate most clearly between the normal and bacteriuria samples had emission wavelength at 425 ± 10 nm and 380 ± 10 nm, with 330 ± 20 nm and 280 ± 5 nm excitation respectively (Fig. 1), an offset wavelength $\Delta\lambda$ of 90 nm was chosen for SFS to enable capturing the fluorescence in these regions of interest.

Fig. 2 shows that undiluted bacteriuria samples have higher fluorescence intensity when excited at 280 ± 5 nm with an emission peak at around 375 ± 10 nm, as compared to undiluted normal urine samples. A two sample *t*-test showed a statistically significant difference between the two groups ($P = 1 \times 10^{-9}$ for 0.05% error). This result indicates that the $\Delta\lambda = 90$ nm offset in SFS can be used effectively to differentiate undiluted bacteriuria samples from normal urine. This result is also in agreement with the higher fluorescence intensity observed for 25 undiluted bacteriuria samples as compared with the 45 normal urine samples when excited at 290 nm (Fig. 3).

3.3. *Effect of dilution of urine and offset $\Delta\lambda$ of 30 nm for synchronous fluorescence spectra (SFS)*

An earlier study [10] advocated diluting the urine sample 1:30 as well as using a $\Delta\lambda$ of 30 nm to characterize a single UTI sample. We tested 25 normal and 25 bacteriuria samples using similar parameters, *i.e.* the urine samples were centrifuged (10 min, 1100 rpm) and diluted 1:30 in double distilled water and the SFS was obtained using a $\Delta\lambda$ of 30 nm.

Contrary to the earlier report [10], no clear difference was observed between the two groups of samples (Fig. 4a and b). However, an offset $\Delta\lambda$ of 90 nm shows a clear distinction between the two groups of diluted samples (Fig. 4c and d), with the ratio of autofluorescence at 280 nm and 325 nm excitation between the groups showing a significant difference ($P = 0.0035$ for 0.05% error).

3.4. *The influence of inner filter effect on the EEMs and SFS (with offset $\Delta\lambda = 90$ nm) of undiluted normal human urine and undiluted bacteriuria samples*

It is well known that the recorded fluorescence intensity may not be proportional to the fluorophore concentration due to a well-

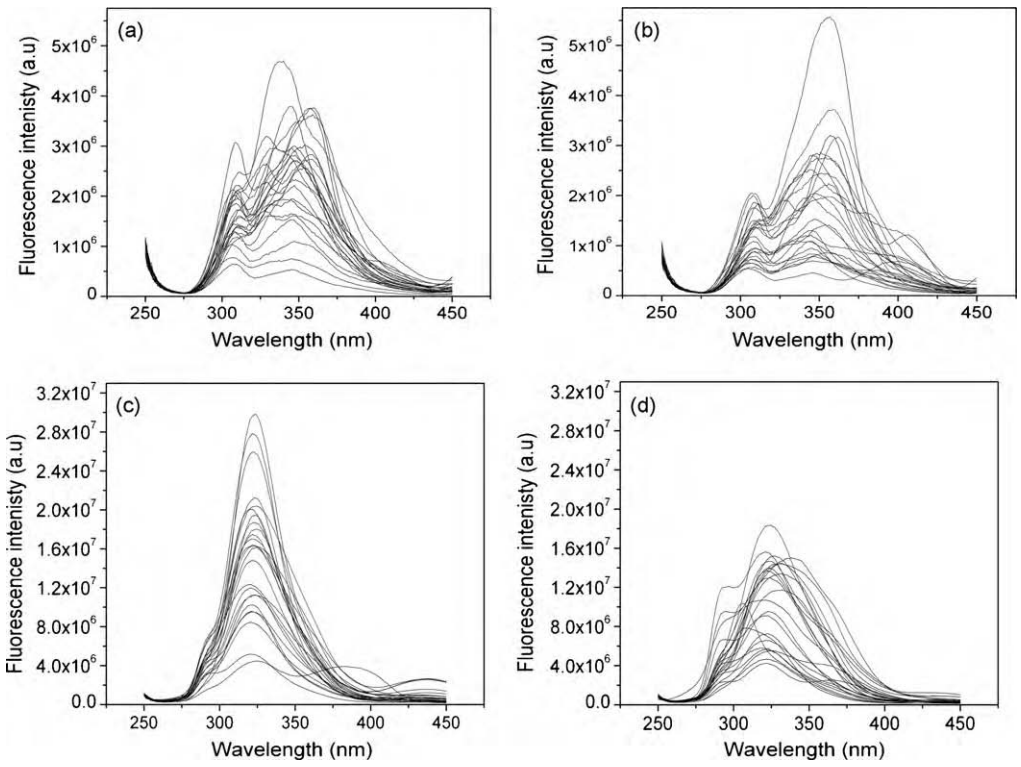

Fig. 4. Comparison of SFS of diluted (1:30) normal human urines with diluted (1:30) bacteriuria samples measured using 10 mm cuvette. (a) and (c) SFS of diluted normal human urine with offset wavelengths $\Delta\lambda = 30$ nm and $\Delta\lambda = 90$ nm respectively; (b) and (d) SFS of diluted bacteriuria samples with offset wavelengths $\Delta\lambda = 30$ nm and $\Delta\lambda = 90$ nm respectively. Each curve represents a different urine sample.

known phenomenon referred to as the inner filter effect [21]. This effect can be due to the absorption of excitation light by the sample (primary inner filter effect) and the reabsorption of emitted light (secondary inner filter effect) [21], or both. These effects significantly distort the spectral range where optical density of the sample is high. In order to understand spectral variations due to the inner filter effect, we also analysed the urine samples by using a shorter path length cuvette. There exist different methods to correct for inner filter effects, which depend, among others, on the geometry of the system (L format, front face, etc.) [22,23]. These corrections not only result in the intensity change at a given wavelength but also lead to the corrections in fluorescence spectrum shape and peak location. We stress that in this work we did not attempt to correct for inner filter effect. This was unnecessary because the objective was to distinguish between healthy and bacteriuria samples and not producing the artefact free spectra of urine.

A comparison of the SFS ($\Delta\lambda = 90$ nm) of undiluted urines measured using a 10 mm (Fig. 2 inset) and a 4 mm (Fig. 5) cuvette clearly showed that the spectral patterns obtained with these cuvettes were different. With the 4 mm cuvette, both undiluted normal and bacteriuria samples showed higher emission intensity in the shorter UV (250–300 nm), relative to the emission in the 300–450 nm range. This feature was not observed for urine samples measured with 10 mm cuvette. This is due to higher inner filter effect in the 10 mm cuvette compared to 4 mm cuvette.

However, Figs. 2 and 5 also show that irrespective of the cuvette dimension (*i.e.* 4 mm or 10 mm), there is higher emission intensity for undiluted bacteriuria samples compared to undiluted normal samples when excited in the shorter UV region (250–300 nm). Further, taking the ratio of emission intensity between 280 nm and 320 nm excitation wavelengths for the samples measured using 4 mm cuvettes clearly differentiated the undiluted normal and bac-

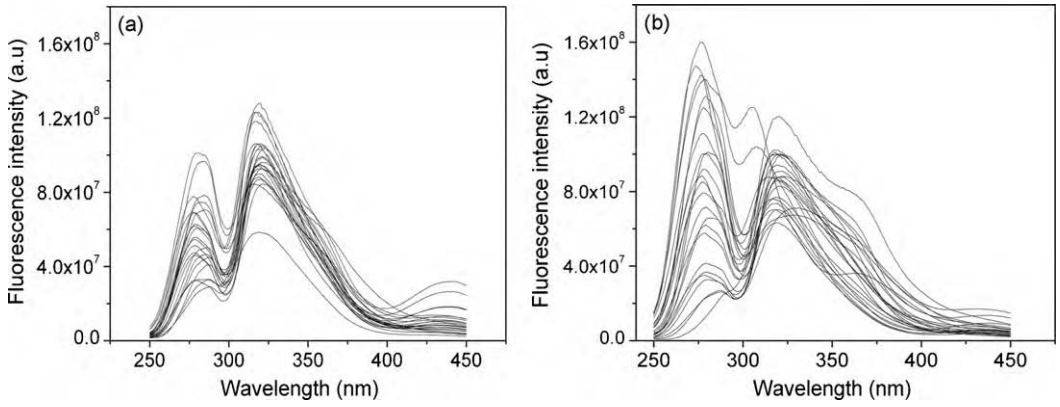

Fig. 5. Comparison of SFS ($\Delta\lambda = 90$ nm) of undiluted normal human urine (a) with undiluted bacteriuria samples and (b) measured in 4 mm cuvette. Each curve represents a different urine sample.

Table 1
Concentration of indoxyl sulfate and tryptophan in urine samples, determined by HPLC.

Sample	Indoxylsulfate concentration (μg/ml)	Tryptophan concentration (μg/ml)
Normal urine samples		
1	76.0	4.1
2	121.6	9.1
3	101.3	8.6
4	159.4	8.0
Mean ± SD	114.5 ± 35.2	7.5 ± 2.2
Bacteriuria samples		
1	150.3	No peak
2	154.4	12.6
3	170.2	7.2
4	156.9	8.0
Mean ± SD	157.9 ± 8.5	9.2 ± 2.9

teriuria samples ($P = 7 \times 10^{-4}$ for 0.05% error). These results show that even though inner filter effect influences the autofluorescence spectrum of undiluted urine, it is still possible to differentiate between undiluted normal and undiluted bacteriuria samples using an offset value of $\Delta\lambda = 90$ nm.

3.5. Comparison of the concentration of tryptophan and indoxyl sulfate in normal human urine and bacteriuria samples

In order to shed some light on the identity of fluorophores responsible for the observed fluorescence intensity variations between UTI and normal urine, we carried out HPLC measurements. This is because the examination of fluorescence EEMs alone is generally not sufficient for chemical analysis of urine.

The fluorophores which can contribute most to the emission peak at 410 ± 10 nm when excited at 280 ± 5 nm include indoxyl sulfate, tryptophan, indolyl-3-acetate and xanthine (Supplemental Material, Appendix 1). The level of these fluorophores in urine may be linked to health conditions such as UTI. Bacteria such as *E. coli* can reduce tryptophan to indole resulting in a variation of the concentration of tryptophan and its metabolites [24] which can alter the fluorescence. In order to test this hypothesis, in the present study, we analysed the concentration of indoxyl sulfate and tryptophan in normal and bacteriuria samples, by HPLC. As seen from Table 1, there is a slightly higher but not significant concentration of indoxyl sulfate in bacteriuria samples when compared to the normal urine samples. It is debatable whether this small concentration difference underpins the higher fluorescence observed for bacteriuria samples. HPLC results also show no significant differences in the concentration of tryptophan between the two sample groups.

Finally we consider other factors contributing to the differences in autofluorescence between normal and bacteriuria samples. It has been reported that in UTI, xanthine oxidase (XO) activity is above 1000 units/l compared to its negligible quantities in sterile human urines [16]. XO catalyses the oxidation of hypoxanthine to xanthine and to uric acid. During this process, super oxides are also generated [25,26]. Xanthine is excited at 270 ± 10 nm with an emission peak around 350 nm [27]. We suggest that the increased autofluorescence of bacteriuria samples excited at shorter UV could be due to the variation in the concentration of xanthine, uric acid and the generation of super oxide in bacteriuria samples due to XO activity.

The fluorophores which could mainly contribute to emission at 425 ± 10 nm at 330 ± 20 nm excitation are 4-pyridoxic acid, 3-hydroxy-anthranilic acid, xanthurenic acid and neopterin (Supplemental Material, Appendix 1). The possibility of 3-hydroxy-anthranilic acid and xanthurenic acid contributing to the differences observed in the fluorescence between the normal and bacteriuria samples is remote, in light of an earlier report that the mean excretion of 3-hydroxy-anthranilic acid and xanthurenic acid for normal and UTI patients are similar [28]. However, patients with bacterial urinary tract infections are known to have increased neopterin concentration in their urine, associated with protracted infection rather than with local activation of immune cascades and direct release of neopterin into the urinary tract [17]. Therefore the spectral blue shift observed in the emission region 425 ± 10 nm with 330 ± 20 nm excitation for the bacteriuria samples could be attributed to such increased neopterin concentration.

3.6. Conclusions

In this study, UV autofluorescence of bacteriuria samples and normal human urine samples were analysed in detail. Even without dilution, pronounced spectral differences were observed in the EEMs in the shorter UV (250–300 nm) region, as well as a clear difference in the emission intensity at 290 nm for the two groups of urine samples. We have also shown that SFS with an offset $\Delta\lambda = 90$ nm can be used effectively to differentiate between undiluted bacteriuria and normal urine samples. We tested the concentration of two key indole compounds (indoxyl sulfate and tryptophan) by using HPLC, in an attempt to explain their role in urine autofluorescence. However, HPLC analysis did not reveal any significant variation in the concentration of tryptophan and indoxyl sulfate in the normal urine and bacteriuria samples. We suggest that multiple factors such as xanthine oxidise activity, tryptophanase and higher neopterin concentration could possibly contribute to the differences observed in the fluorescence spectra between the two groups of urine samples.

Our results provide a proof of the concept for using the fluorescence spectrum of urine as a diagnostic tool to differentiate between normal and UTI urine, despite strong physiological variability between individual samples.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2010.05.049.

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