Fluorescence characteristics of human urine from normal individuals and ovarian cancer patients

M. ZVARIK[1,*], D. MARTINICKY[2], L. HUNAKOVA[3], I. LAJDOVA[4], L. SIKUROVA[1]

[1]*Department of Nuclear Physics and Biophysics, Faculty of Mathematics, Physics and Computer Science, Comenius University, Bratislava, Slovakia;* [2]*Department of Gynecological Oncology, National Cancer Institute, Bratislava, Slovakia;* [3]*Cancer Research Institute, Slovak Academy of Sciences, Bratislava, Slovakia;* [4]*Department of Clinical and Experimental Pharmacology, Faculty of Medicine, Slovak Medical University, Bratislava, Slovakia*

*Correspondence: zvarikmilan@gmail.com

Early diagnostics of ovarian cancer is difficult, because there are no symptoms until the disease has progressed to an advanced stage. As urine contains many intrinsic fluorophores, modern fluorescence techniques are perspective candidates for new routine urine tests. The presented work deals with differences in the fluorescence of metabolites in urine of ovarian cancer patients comparing to healthy volunteers using the fluorescence excitation-emission matrices. The most serious differences were found in undiluted urine at the fluorescence emission wavelengths from 400 nm to 460 nm when excited at 310 – 390 nm. Statistical analyses of our data have shown a 5-fold reduction in the intensity of the peak at 330/420 nm (excitation/emission wavelength) for undiluted urine samples excreted by cancer patients as compared to those of normal donors. Moreover, the ratio of intensities of the peaks at 370/440 nm and at 330/420 nm is 18-times elevated in urine excreted by patients with ovarian cancer as compared to healthy urine samples. The observed changes could be interpreted as reduction of the presence of pyridoxic acid, whereas blue-fluorescing pteridines becomes dominant in excitation-emission matrices of cancer urine samples in comparison to healthy donors. We suggest pteridines, which are related to cellular metabolism, as suitable candidates for neoplasia-associated fluorescent markers in human urine. Our work showed that monitoring of human urine fluorescent metabolites offers an alternative for ovarian cancer screening.

Key words: urine, fluorescence, ovarian cancer, pteridine

Human urine is a complex biological fluid containing a range of chemical compounds produced by the body. Urine is an aqueous solution of metabolites, inorganic salts and organic compounds. The composition of urine is influenced by many factors including body metabolism, dietary intake, age, hydration and diseases [1]. Analysis of urine is therefore widely used for detection of various health conditions. Standard urine examination includes physical, biochemical and microbiological tests. These analyses require additional slow and costly processes (isolation, purification, etc.). As urine contains many intrinsic fluorophores, modern fluorescence techniques are perspective candidates for new routine urine tests. When compared to other methods, fluorescence analysis is fast, safe, highly sensitive, and noninvasive. Also, this method is stressless for patients and cheap enough to be provided routinely. Fluorescence spectra of urine from healthy humans were first presented by Leiner et al. [2] at various excitation and emission wavelengths in the form of contour plots of excitation-emission matrices, along with tentative assignments of fluorescent peaks. Data presented in [2] have shown that human urine contains a variety of components with strongly overlapping fluorescence bands and can be adequately studied by three-dimensional plot methods. Application of the advanced fluorescence techniques to urine autofluorescence analysis offers a chance to detect urinary metabolites potentially associated with neoplastic process, which could provide a new direction in current search for predictive and prognostic markers [3, 4].

So far, there is only a single study focused on spectral analysis of urine autofluorescence in patients with mixed histological types of tumors [5]. The authors used simple fluorescence emission spectra and Stokes shift spectra to analyze the native fluorescence of urine samples of healthy controls and those of patients with different type of cancer.

In this study, we concentrated on the analysis of changes in urine autofluorescence from patients with ovarian cancer

in comparison to healthy subjects. The changes in intrinsic fluorescence were studied by three-dimensional plot methods of fluorescence excitation-emission matrices, where the characteristic circular patterns resulting from fluorescence were used to identify particular wavelengths suitable for proper selection of fluorophores. The aim of the study was to investigate the possible cancer screening potential of human urine autofluorescence.

Materials and methods

This study has been cleared by The National Cancer Institute (Slovakia) Ethics Review Board for human study and registered with the number FAM OVCA 1. The research was carried out according to the World Medical Association Declaration of Helsinki and patients have signed an informed consent.

32 morning urine samples from fasting normal volunteers and 37 oncological patients were used in this study. All of them were analyzed for pH, protein, glucose, bilirubin, nitrate, hemoglobin, ketones, acetone, and urobilinogen. The presence of red blood cells, white blood cells, casts, epithelial cells and crystals was also tested in these samples at the Department of Clinical Biochemistry, National Cancer Institute, Bratislava, Slovakia. All of 32 samples from volunteers (women aged 36 - 73) showed no abnormal laboratory findings and they were referred to healthy samples, i.e. controls. 37 urine samples were taken from oncological patients (women aged 36 – 78) before radical surgery for ovarian cancer. The histological examination of removed tissues confirmed ovarian malignant tumors (ICD 10 code C56). Urine samples were taken before the start of anticancer or antibiotic therapy. Urine samples were centrifuged at 3000 rpm for 10 min at room temperature (22 ± 1ºC) and undiluted supernatants were used for spectral analysis. The detailed fluorescence data for excitation-emission matrices (EEMs) of urine samples were obtained on a LS45 (PerkinElmer) luminescence spectrometer using the FL WinLab software. Measurements were taken at room temperature (22 ± 1ºC) in an ultra micro quartz cuvette (5 mm excitation path length and 1 mm emission path length). Both emission and excitation slit widths were set to 10 nm. Fluorescence emission spectra were taken at excitation wavelengths from 250 to 530 nm in intervals of 20 nm. Emission was taken in intervals of 270 - 650 nm. Excitation-emission matrices represent the relation among the fluorescence intensity, excitation wavelength and emission wavelength. They were created of the set of emission fluorescence spectra of urine samples with various excitation wavelengths. The x-axis represents the emission wavelength and the y-axis the excitation wavelength. Contour lines connect regions of the same fluorescence intensity and each contour line represents 5 percent of the full fluorescence scale. The collected data were graphically and statistically processed by the R software. The Mann–Whitney *U* test was used to compare mean values of fluorescence of urine samples from healthy and oncological patients.

Results

Typical fluorescence emission spectra of urine from healthy humans are presented in Fig. 1a) as contour plots of excitation-emission matrix (EEM) for undiluted urine. We can resolve three main bands in the EEMs: (A) at 450/520 nm (peak excitation/peak emission), (B) at 370/520 nm and (C) with a maximum at 330/420 nm and a shoulder at 370/440 nm (Fig. 1a). All of these excitation-emission characteristics were found in each of the 32 urine samples from healthy controls.

The EEMs of urine from cancer patients were different from the EEMs from healthy volunteers. Although we observed similar spectral characteristics of the peaks (A) at 450/520 nm and (B) at 370/520 nm in urine samples from cancer patients (Fig. 1b), the (C) band exhibits visible differences both in the peak position and fluorescence intensity. Whereas fluorescence of the (C) band in healthy volunteers is maximal at 330/420 nm with only a shoulder at 370/440 nm, urine samples from oncological patients are characterized by pronounced depression of fluorescence at 330/420 nm and domination of the peak at 370/440 nm in this spectral region. As a result, we can see a shift from 330/420 nm to 370/440 nm in the (C) band position. Moreover, urine samples of cancer patients can be described by weak fluorescence in the whole excitation-emission region of the broad (C) band.

The information obtained from detailed contour plots of EEMs enabled us to identify a 5-fold reduced intensity of the peak at 330/420 nm for undiluted urine samples from cancer patients as compared to controls (Fig. 2a). Moreover, the ratio of intensities of the peaks at 370/440 nm and at 330/420 nm is 18-times elevated in patients with ovarian cancer compared to healthy persons (Fig. 2b). On the contrary, the intensities at 450/520 nm, do not show any significant changes (Fig. 2a).

Discussion

Three fluorescent bands were recognizable in the EEMs obtained from undiluted urine samples of both healthy (Fig. 1a) and cancer (Fig. 1b) subjects involved in our research. We labeled the observed bands as A, B, C.

The (C) band could be composed of several emission peaks and we are able to distinguish at least two peaks at 330/420 nm and at 370/440 nm. The peak at 330/420 nm can be attributed to several fluorophores; pyridoxic acid is the strongest fluorescent species from this group and other urine compounds, such as uric acid, xantine or hydroxyanthranilic acid [6] might contribute to this fluorescence, too. The peak at around 370/440 nm may be formed by one or more species, mainly blue-fluorescing pteridines, and perhaps kynurenines [2, 3]. Tissue fluorescence at 440 nm can be caused also by collagen and NADH, predominantly bound to a protein [5], but it is difficult to envisage the presence of these molecules in healthy urine samples [7]. The peaks (A) and (B), emitted at 520 nm (Fig. 1a) are most likely due to the presence of flavins and their metabolites, which are normally found in urine at
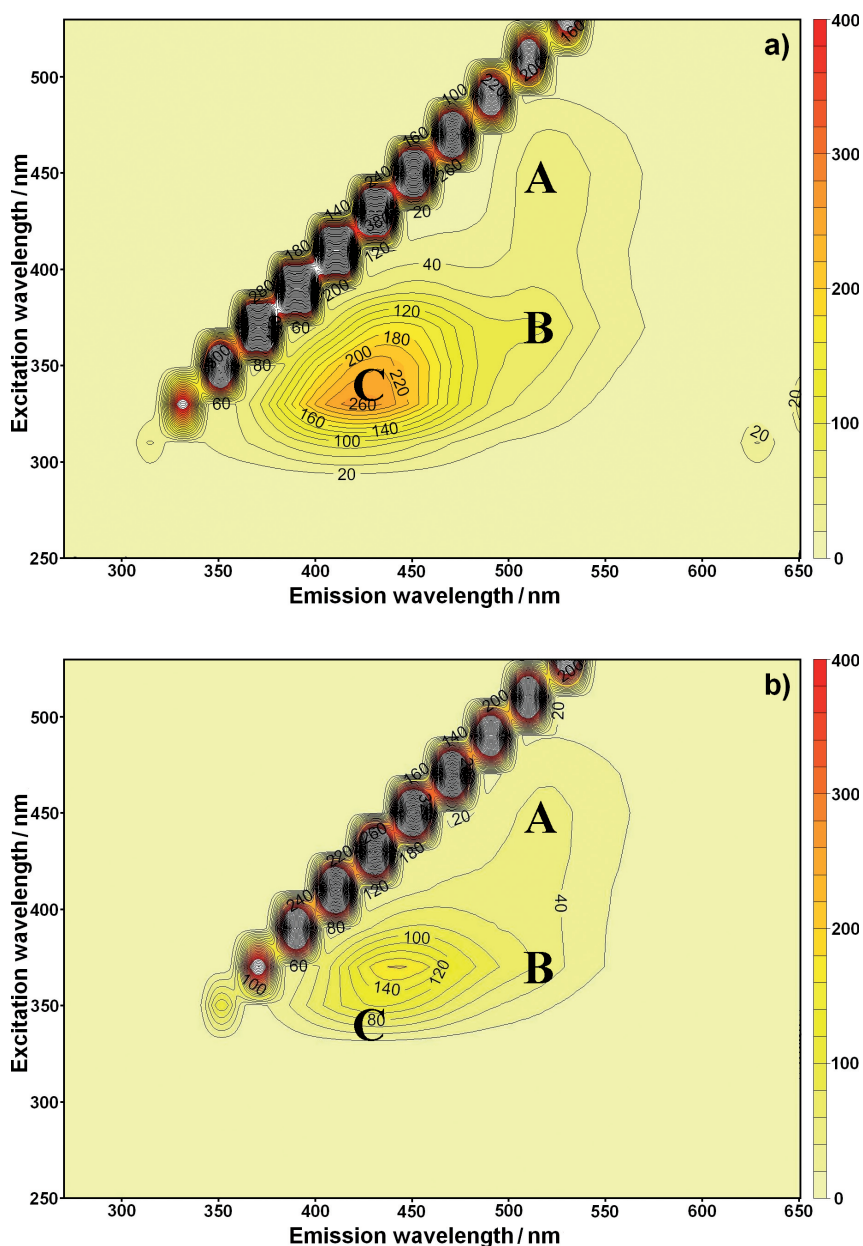
Figure 1. Excitation-emission matrix of a representative human urine sample from a) a healthy person and b) an oncological patient. Contour lines connect regions of the same fluorescence intensity and each contour line represents 5 percent of the full fluorescence scale. A, B, C - indicate the location of the observed excitation – emission maxima.

low concentrations (2 nmol/ml) [2, 8]. Fluorescence spectra of flavins are characterized by two excitation wavelengths at about 370 nm and 440 nm [9]. Also bilirubin could contribute to the peak (A). However, bilirubin emits a considerably lower fluorescence than flavins and we have not seen abnormally elevated bilirubin in our urine samples. Therefore, the contribution of bilirubin to the fluorescence in the investigated samples is most likely negligible. The peak (B) at about 370/520 nm seems to be only a shoulder of the band (C) in EEMs (Fig. 1), thus its

intensity is influenced by the strong fluorescence of the (C) band. Therefore, the peak (B) is not suitable for fluorescence determination of flavins in urine samples.

The comparison of EEMs of undiluted urine samples from patients with ovarian cancer and from healthy subjects demonstrates that the most serious differences have occurred in the spectral region of the (C) band, i.e. in the excitation wavelength range of 310 – 390 nm and the emission interval of 400 - 460 nm. Apparently, urine samples from cancer
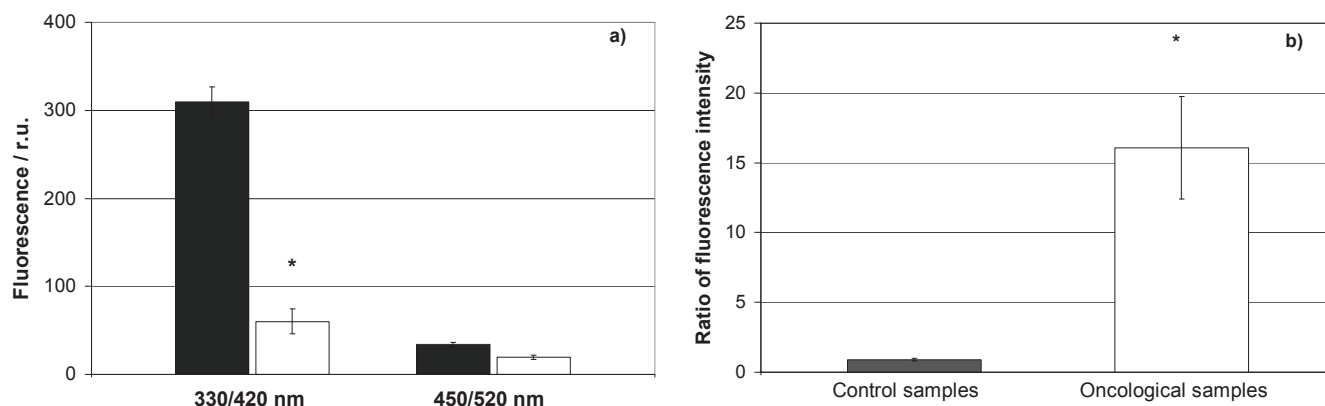
Figure 2 a) The values of fluorescence intensity at 330/420 nm (peak excitation/emission wavelengths) and at 450/520 nm and b) the ratio of fluorescence intensities of the peaks at 370/440 nm and at 330/420 nm for control (black bars, n = 32) and oncological (white bars, n = 37) urine samples. Data are expressed as average ± SEM. * – significant difference at level p < 0.001 between the control and oncological groups.

patients possess weaker fluorescence in the whole excitation-emission region of the broad band (C) in comparison with controls. Similar results were also reported by Masilamani et al. [5]. Moreover, statistical analysis of our data revealed a 5-fold reduction (p < 0.001) in the intensity of peak (C) at 330/420 nm (Fig. 2a) for undiluted urine samples from cancer patients compared to normal donors. Fading of the peak at 330/420 nm in cancer urine samples is accompanied by emergence of a peak at 370/440 nm that becomes dominant in the EEMs of urine samples in cancer patients, whereas it was manifested only as a shoulder to the intensive maximum at 330/420 nm in the EEMs of healthy subjects. Correspondingly, the ratio of intensities of the peaks at 370/440 nm and at 330/420 nm is elevated 18 times in urine from ovarian cancer patients compared to healthy volunteers (Fig. 2b). This means that urine samples from oncological patients are characterized by dominance of the peak at 370/440 nm and pronounced depression of fluorescence at 330/420 nm as compared to controls. As a result, we can see a red shift in wavelengths of the band (C) maximum from 330/420 nm in normal samples to 370/440 nm in urine samples of patients with ovarian cancer. The revealed red spectral shift makes this characteristic well resolvable. Observed changes in the (C) band can be most likely interpreted as significant reduction, even disappearance of pyridoxic acid [2], whereas the blue-fluorescing pteridines [2] become dominant in EEMs of cancer urine samples.

Pyridoxic acid is the catabolic product of vitamin B6, which is excreted in the urine. Several forms of vitamin B6 are known, but pyridoxal – phosphate is the principal active form of vitamin B6 that is involved in almost 100 enzymatic reactions. Deficiency of vitamin B6 has been associated with considerably impaired carbon metabolism. Hence, low vitamin B6 levels may increase cancer risk. Larsson et al. have found that vitamin B6 may reduce risk of colorectal cancer as well as ovarian cancer [10, 11].

Pteridines have become a focal point of cancer screening research in the last two decades because certain pteridine levels have been shown to reflect the presence of cancer [12 - 14]. Pteridines also play important roles in the synthesis of some vitamins, and are intermediates in anabolic and catabolic reactions and serve as coenzymes mediating the action of some enzymes. Malignant tumors possibly alter the biosynthesis and metabolism of pteridines, thus leading to a change in pteridine concentrations [13]. The blue fluorescing derivatives of these compounds include pterins and folates. Although folate is essential to numerous bodily functions, its fluorescence quantum yield is many times lower as compared to those of pterins [15]. The most important blue-fluorescing pterins excreted in urine are neopterin, biopterin, xanthopterin, isoxanthopterin, and pterin. Pterins can exist in different oxidation stages, but only fully oxidized pterins are highly fluorescent, while the fluorescence of the reduced derivatives is much weaker. Rokos et al. [12] presented data that indicated higher concentrations of urine neopterin in patients with non-Hodgkin's lymphoma (7 cases) or with liver metastases (12 cases) then in control subjects. Han et al. [13] showed that the levels of pterins were found to be significantly elevated in urine excreted by cancer patients (9), without specifying cancer type. Gamagedara et al. [14] found that the levels of pterins were higher in samples from cancer patients than in those from healthy subjects. The urine samples from cancer patients represented various combinations of breast (12), lung (9), colon (4), rectal (2), pancreatic (1), ovarian (3), esophageal (4), bladder (1) and kidney (1) cancers and non-Hodgkin lymphomas (4).

In our study we focused exclusively on ovarian cancer and we compared female subjects, both with cancer and healthy. Our results are statistically highly significant (p < 0.001) and suggest that fluorescence features of urine are worth of pursuing for the further biomedical applications. However, to elucidate the etiopathology of observed changes, more detailed research is needed and is in progress.

Conclusions

In this study, we explored the differences in fluorescence characteristics of human urine between healthy subjects and patients with ovarian cancer. The detailed fluorescence data in the form of 3D contour plots of excitation-emission matrices, scanned at excitation wavelength range from 250 to 530 nm and emission taken in intervals of 270 - 650 nm were successfully used for quantitative analysis of undiluted urine. Our data have revealed important differences at the emission wavelengths from 400 nm to 460 nm when excited at 310 – 390 nm, both in the peak position and fluorescence intensity. Healthy urine fluorescence is maximal at 330/420 nm with only a shoulder at 370/440 nm, whereas urine samples from cancer patients are characterized by pronounced depression of fluorescence at 330/420 nm and dominance of the peak at 370/440 nm in this spectral region. As a result, we can see a red shift from 330/420 nm to 370/440 nm. Statistical analyses revealed a 5-times reduction ($p < 0.001$) in the intensity of the peak at 330/420 nm for urine samples from cancer patients as compared to those of normal donors. Ratio of intensities of the peaks at 370/440 nm and at 330/420 nm is approximately 18-times elevated in the urine of ovarian cancer patients compared to healthy urine samples. The observed changes can be interpreted as a reduction of pyridoxic acid fluorescence, whereas blue-fluorescing pteridines become dominant in excitation-emission matrices of urine samples from cancer patients in comparison to healthy donors. On the contrary, the intensities at 450/520 nm, corresponding to flavins, did not show any significant changes.

Thus we showed that monitoring of human urine autofluorescence offers an alternative for ovarian cancer screening. According to our findings, we suggest pteridines, whose presence in urine reflects changes in cellular metabolism, as suitable candidates for neoplasia-associated fluorescent markers in human urine.

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