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ORIGINAL PAPER

Synchronous Luminescence Spectroscopic Characterization of Urine of Normal Subjects and Cancer Patients

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Abstract Urine is one of the diagnostically potential bio fluids, as it contains many metabolites and some of them are native fluorophores. These fluorophores distribution and the physiochemical properties may vary during any metabolic change or at different pathologic conditions. Since urine is a multicomponent fluid, synchronous luminescence technique, a powerful tool has been adopted to analyse multicomponents in single spectrum and to resolve emission spectrum without much of photobleaching of fluorophores. In this study, urine samples of both normal subjects and cancer patients were characterised using synchronous luminescence spectroscopy with a Stokes shift of 20 nm. Different ratio parameters were calculated from the intensity values of the synchronous luminescence spectra and they were used as input variables for a multiple linear discriminant analysis across normal and cancer groups. The stepwise linear discriminant analysis classifies 90.3 % of the original grouped cases and 88.6 % of the cross-validated grouped cases correctly.

Keywords Synchronous luminescence spectroscopy · Urine · Pteridine · Riboflavin

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Introduction

Native fluorescence spectroscopy of tissues and bio fluids with real time evaluation has been considered as one of the potential methods for monitoring the minor changes in the structure and microenvironment of the native fluorophores [1–4]. The photophysical properties of native fluorophores such as indoxyl sulphate, pteridines and its derivatives, reduced nicotinamide adenine dinucleotide (NADH), riboflavin, collagen, elastin and their cross links, the aromatic amino acids and porphyrins have been considered as a measure to understand the alterations in the functional, morphological and microenvironmental changes in cells and tissues [4].

Based on the report of Alfano and his group on the detection of dental carries and subsequently cancer using native fluorescence, the field of optical biopsy has been given importance in diagnostic oncology [2, 5]. In this context, many reported the characterisation and discrimination of various neoplastic conditions of cells and tissues from their normal counterpart using steady state native fluorescence spectroscopy [6–9]. However, only limited studies are available on the characterization of biofluids using native fluorescence spectroscopy in diagnostic oncology. Among various biofluids, urine has been considered to be one of the diagnostically important biological fluids as it has many native fluorescing metabolites [10]. Studies reported that there was a considerable variation in the concentration and conformation of some of the metabolites due to some altered metabolic and pathological conditions [11].

Among various fluorescence spectroscopic techniques, the synchronous luminescence (SL), also referred as Stokes shift spectroscopy (SSS) technique provides highly resolved spectra of the endogenous fluorophores even in complex systems [12–16]. Although many reported extensively, the use of fluorescence spectroscopy in the discrimination of different

pathological conditions of tissue from their normal counterpart, only limited reports are available to understand the reasons for the altered spectral signature between normal and abnormal cells and tissues [17, 18]. In particular, the application of synchronous luminescence spectroscopy of biofluids in diagnostic oncology is still in the primitive stage [14, 19–22].

This is because, SL technique has to be optimised during the analysis of multicomponents of fluorophores present in urine and other biofluids. The intensity of peaks and resolution of the peaks depends on the scanning wavelength interval ($\Delta\lambda$) between the excitation and emission monochromators. In this context, it is worth to mention that the analysis of urine by SL technique and the optimization of $\Delta\lambda$ is not yet fully understood. The synchronous technique allows the stronger peaks to be increased by using a suitable $\Delta\lambda$. Several groups have reported the use of synchronous luminescence spectroscopy (SLS) as a diagnostic tool with different $\Delta\lambda$ values. For example, Dubayova et al., reported on the use of synchronous fluorescence spectroscopy for diagnostic monitoring of urine with $\Delta\lambda$=30 nm [19]. Masilamani et al., utilised synchronous fluorescence with 70 nm as wavelength offset in the diagnosis of cancer [20]. Sandeep et al., reported the use of 30 and 90 nm as $\Delta\lambda$ for measuring the fluorescence of urine in the diagnosis of human urinary tract infection [21]. Wan et al., made use of synchronous fluorescence spectroscopy with 65 nm as $\Delta\lambda$ and reported the variation of pteridine and its derivatives levels is significant in the urine of stomach cancer patients when compared to the normal subjects [22]. Recently, Yang Pu et al., highlights the importance of optimisation of Stokes shift spectroscopy in cancer diagnosis [15]. Based on these, attempts were made to study the SL characteristics of urine samples of cancer patients and normal subjects. Linear discriminant analysis and receiver operator characteristics based on the intensity ratio variables of the synchronous luminescence spectral data were carried out to discriminate the cancer patients from that of the normal subjects.

Materials and Methods

Samples

First voided morning urine samples were collected in a sterile container from cancerous patients of different aetiologies and stages, who are awaiting for treatment in various hospitals in Chennai and from healthy volunteers. Ethical clearance was obtained from Health and Welfare Department, Government of Tamilnadu, India. As a total of 90 normal subjects both male and female in the age group of 20 to 65 years and 85 pathologically confirmed cancerous patients [of which 40 from oral cancer, 45 from cervical cancer] in the age group of 20 to 70 years. Both the cases are selected in such a way that they are free from any abnormalities like diabetes, jaundice, and bacterial infections. The urine samples were stored in the refrigerator at 4 °C and were examined as such after thawing to room temperature within 48 h of sample collection.

Chemicals

Neopterin and Riboflavin were obtained from Sigma-Aldrich Co. (St. Louis, MO). Stock solutions were prepared using deionised water and were used as standards of fluorescence analysis.

Synchronous Luminescence Measurements

The synchronous luminescence spectroscopic (SLS) characterisation of undiluted urine samples were measured at room temperature using a commercially available spectrofluorometer [Fluoromax-2 SPEX, Edison, New Jersey]. During data acquisition, the excitation and the emission monochromators were simultaneously scanned with a constant wavelength difference between them and the SL signal was collected in the range 250 to 600 nm. The SL spectra measurements were tried with different $\Delta\lambda$ values ranging from 10 to 90 nm with an increment of 10 nm. Among all the $\Delta\lambda$ values, $\Delta\lambda$=20 nm showed better profile and was set as an optimal offset value for the entire experiment. Excitation and emission slit width were fixed as 5 nm. The acquisition interval and the integration time were maintained as 1 nm and 0.1 s respectively.

Statistical Analysis

Detailed statistical analysis of the native fluorescence emission spectral data at $\Delta\lambda$=20 nm excitation was carried out. This includes the following three primary steps. 1. Normalisation of each fluorescence spectrum; 2. Identifying characteristic spectral features of each experimental group and introduction of different ratio parameters and 3. Classification by receiver operator characteristic analysis and stepwise multiple linear discriminant analysis using SPSS/PC+ 15 software [4, 23, 24]. As the present study is aimed to discriminate the malignant subjects from the normal subjects, the analysis was performed across ninety normal subjects and 85 patients with cancers. The protocol for the statistical analysis is the same as that of our earlier work [24].

Results

SLS Characterization of Urine Samples of Normal Subjects and Cancer Patients

The averaged and normalised synchronous luminescence spectra of urine samples of normal subjects and cancer

patients are shown in Fig. 1a and b. The averaged synchronous luminescence spectra show considerable differences between the normal and the cancer patients. From Fig. 1a it is observed that the SL spectrum of normal urine sample show a prominent maximum around 369 nm, whereas in the case of cancer patients, it is centered around 483 nm. The absolute fluorescence intensity at 369 nm is almost the same for both normal subjects and cancer patients. However at 483 nm, the intensity is significantly higher for cancer patients than that of normal subjects. It is observed that both normal and cancer samples exhibits peaks around 369 nm, 393 nm, 483 nm and humps around 420 and 437 nm. A small hump around 515 is observed only in the case of normal subjects. In the case of the cancer spectra, a markable peak at 450 nm is not observed. This may be due to the overwhelming effect of the 480 nm peak and also 450 nm falls in the foothill region of the 480 nm peak. Figure 2a and b shows the averaged and normalised SL spectra of urine of normal and patients with different types of

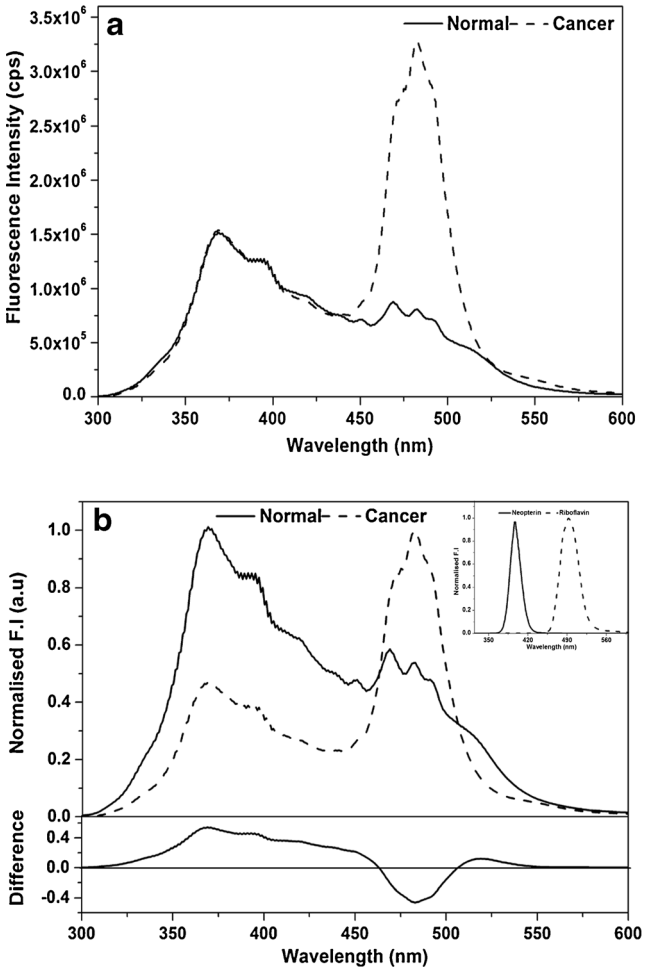

Fig. 1 Averaged and normalised synchronous luminescence spectra of urine of normal subjects and cancer patients. **a** absolute spectra and **b** normalised spectra along with the difference between them and the synchronous luminescence spectrum of standard fluorophores neopterin and riboflavin as inset

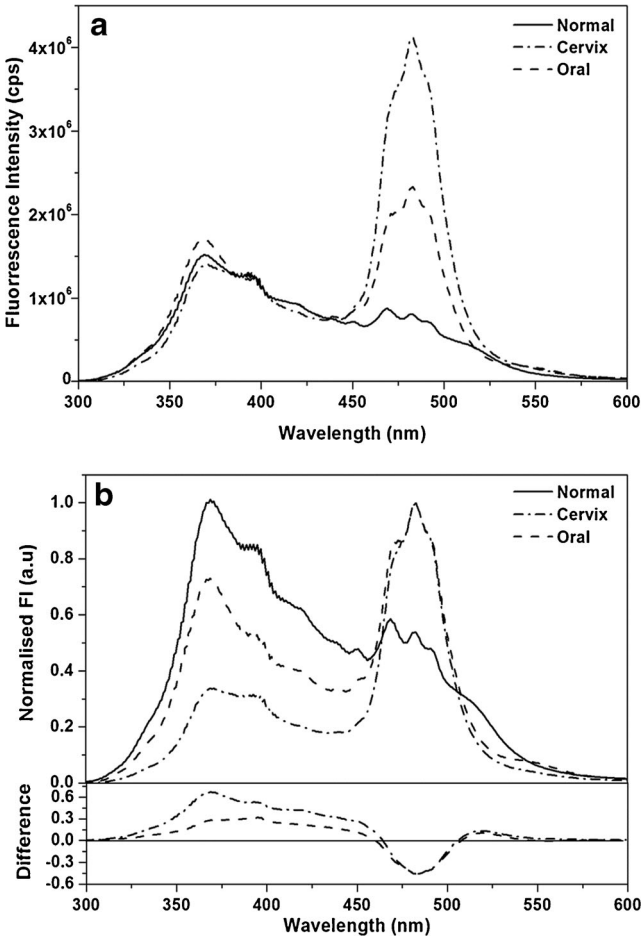

Fig. 2 Averaged and normalised synchronous luminescence spectra of urine of normal subjects and cancer patients of different origins. **a** absolute spectra and **b** normalised spectra along with the difference between them

cancer. From this figure, it is observed that the fluorescence emission intensity around 369 nm varies in the order of oral cancer > normal > cervical cancer whereas around 480 nm it is in the order of cervical cancer > oral cancer > normal subjects.

Further to understand the spectral difference between the normal subjects and patients with cancer, difference spectra was computed by subtracting the normalised spectral intensity of cancer patients from the normal subjects. From Fig. 1b, it is found that, the difference spectrum between normal subjects and cancer patients has two positive regions around 370 and 515 nm and a negative region around 480 nm. From the difference spectrum, it is observed that the luminescence intensity of cancer patients is higher around 480 nm than that of normal subjects. In the other two regions viz. 370 and 515 nm the normal subjects have higher intensity. From Fig. 2b, same results were observed from the difference spectrum of normal subjects and patients with different types of cancer. However, oral cancer shows much difference than cervical cancer in the 370 nm region with respect to normal subjects. From the difference spectrum Fig. 1b, it is observed

that the difference is positive in the region between 340 to 460 nm and 500 and 535 nm. It is also observed that the difference is negative between 460 and 500 nm. This clearly indicates that there is a considerable variation between normal and samples with abnormality

Results of Statistical Analysis

To quantify the observed spectral differences between normal and cancerous subjects, 10 ratio variables were introduced from luminescence intensities at different wavelengths and were introduced in the analysis. The mean with standard error values of these ratio variables for normal and diseased group of samples studied are given in Table 1 along with their significance (P) values. The results of the stepwise multiple linear discriminant analysis and ROC performed are given below.

Discrimination by Stepwise Multiple Linear Discriminant Analysis

The stepwise linear discriminant analysis was performed across ninety normal and 85 cancerous samples resulted in the following expression for a canonical discriminant function (DF):

$$DF = -[0.087^{*}\ V_2] + [3.544\ ^{*}V_4] - [0.165^{*}\ V_5] - [0.059^{*}\ V_{10}] - [1.037] \quad (1)$$

It is seen from the above expression for canonical discriminant function, out of 10 ratio variables, only four ratio variables turned out to be significant and were included in the linear discriminant function. Figure 3 shows the scatter plot of the discriminant score.

Table 1 Mean values (±SD) of the ratio parameters used for statistical analysis along with their significance (P) values

Parameter	Normal	Cancer	Significance
I_{370}/I_{485} [V_1]	2.75±1.84	1.04±1.55	0.000
I_{397}/I_{485} [V_2]	2.00±1.23	0.67±0.56	0.000
I_{420}/I_{450} [V_3]	1.35±0.35	1.13±0.38	0.000
I_{420}/I_{485} [V_4]	1.39±0.60	0.45±0.33	0.000
I_{420}/I_{522} [V_5]	4.69±2.51	3.57±1.88	0.001
I_{440}/I_{450} [V_6]	1.04±0.12	0.89±0.16	0.000
I_{440}/I_{485} [V_7]	1.08±0.41	0.38±0.35	0.000
I_{440}/I_{522} [V_8]	3.66±2.01	2.86±1.91	0.008
I_{450}/I_{485} [V_9]	1.04±0.37	0.40±0.31	0.000
I_{485}/I_{522} [V_{10}]	3.44±1.43	10.91±6.14	0.000

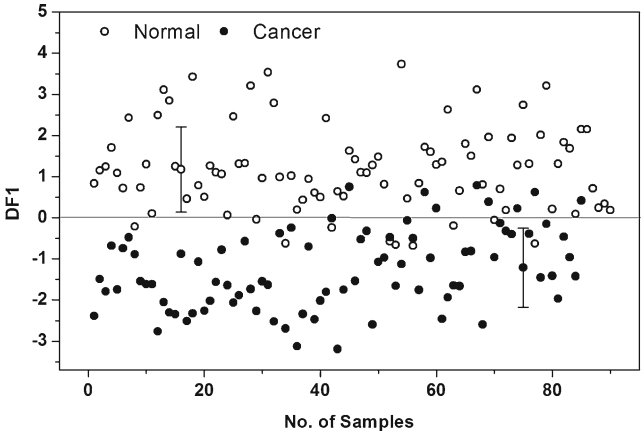

Fig. 3 Scatter plot showing the distribution of DF for normal [*empty circle*] and cancer [*filled circle*] subjects

Discrimination by Receiver Operator Characteristic Curve

Figure 4 shows the ROC graph for the ten ratio variables. The cut off values for each ratio variable and their respective sensitivity and specificity values are given in Table 3. From Table 3, it is observed that the area under curve for the variables V_2, V_4, V_7 and V_9 are closer to 1 [≈above 0.9]. Among these V_9 provides 95.6 % sensitivity and 80.0 % specificity.

Discussion

Though there is tremendous development in the diagnostic and treatment modalities, cancer remains as a major threat to the public. In this context, many showed interest in developing a fast and simple diagnostic method to diagnose the disease prognosis in its early stage as it may reduce patient morbidity and mortality. Based on this, several reports have

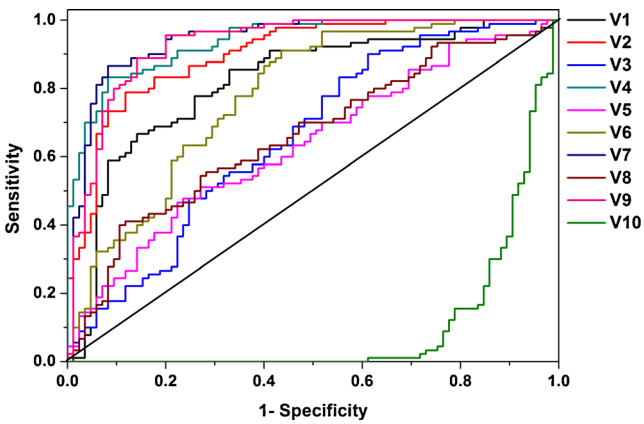

Fig. 4 ROC curves for the ratio variables used in the discriminant analysis. The optimum cut-off point was defined as the closest point on the ROC curve to the point [X,Y] = [0, 1], where X = 1—specificity and Y = sensitivity

been reported on the native fluorescence of tissues and cells [1, 2, 9, 16, 25–27]. However, only limited studies are reported on body fluids [4, 24, 28, 29]. Urine is considered as an important bio fluid as it has many metabolites and it may reflect the metabolic changes in the body [3, 5]. In this context, researchers showed interest in characterising urine towards disease diagnosis [21, 30]. However, the native fluorescence characterisation of urine in clinical oncology is still in its primitive stage. For example, Rabinwitz considered the variation in the red and blue fluorescence emission intensity of urine in differentiating benign and malignant patients with respect to normal counterpart [31]. Yi-Qun Wan made use of synchronous fluorescence spectroscopy and reported the variation of isoxantopterin levels is significant in the urine of stomach cancer patients when compared to the normal subjects [22]. Masilamani et al., and subsequently our group reported the detection of cancer by native fluorescence of urine [14, 20, 24, 28]. However, the use of native fluorescence in differentiating different aetiologies and stages of cancer was not discussed in detail. In this context, attempts were made to characterise the urine by synchronous luminescence spectroscopy and the spectral data were subjected to statistical analysis to check the diagnostic potential of the synchronous luminescence spectroscopy.

Figure 1a and b shows the averaged and normalised synchronous luminescence spectrum of normal and cancer subjects. From Fig. 1a and b, it is observed that the normal subjects exhibits a prominent maximum at 369 nm and small hump around 390 nm which may be attributed to pteridine and its derivatives and small humps around 420 and 437 nm which are mainly due to riboflavin and its metabolites [22]. On the other hand, cancer subjects exhibits the major peak around 483 nm which may be attributed to riboflavin. The small humps around 390, 420 and 437 nm are not clear in the case of cancer subjects when compared to normal subjects which may be due to the overwhelming effect of flavin. In addition to that, in the case of normal subjects a small hump was observed at 515 nm which is mainly due to porphyrin.

The observed blue shift of flavin emission in cancer urine at 483 nm instead of 515 nm of normal subjects may be due to the accumulation of positive ions and the influence of its microenvironment [1]. From Fig. 2a, it is observed that SL intensity of pteridine varies in the order of oral cancer > normal Subjects > cervical cancer.

Generally, riboflavin, pteridine and its derivatives are very important cofactors of cell metabolism and participate in various redox reactions and energy metabolism [22, 32–36]. During the turnover of normal into cancer state, the interlocking coordination of the metabolism may be affected and this may influence the variation in the concentration of the fluorophores in the blood plasma [37]. The fluorophores pteridine and flavin that is not bound to proteins in the plasma is filtered by glomerulus and excreted in urine [38].

Table 2 Classification results of the discriminant analysis

Cases	Actual group	Predicted group membership		Total % of correctly classified cases
		Normal	cancer	
Original	Normal	**91.1**	8.9	90.3
	Cancer	10.6	**89.4**	
Cross-validated	Normal	**88.9**	11.1	88.6
	Cancer	11.8	**88.2**	

[a] Bold figures represent the specificity or sensitivity value of the corresponding group

Cancer cell utilises more riboflavin for energy metabolism than the normal do [36]. During the unfolding of flavo protein the secondary and tertiary structure of the protein may be disrupted and interactions with flavin break down, usually leading to the increase of flavin in blood plasma [39]. This is one of the reasons for more fluorescence in urine as it has more free riboflavin. From Fig. 2a, it is observed that SL of flavin varies in the order of cervical cancer > oral cancer > normal subjects. The variation in the flavin emission may be due to variation in flavin excretion. However, the relation of riboflavin with cancer is very complex. Some have reported that the higher intake of flavin reduces the chances of cancer and some others reported that the deficiency of flavin leads to the growth of cancer [40].

Also, it has been reported that an elevation of pteridine and its derivatives especially neopterin in the urine of cancer patients is significantly associated with the disease progression [32]. From Fig. 2a, it is observed that the oral cancer patients have a slight increase in neopterin excretion than normal subjects which is in good agreement with earlier reports. Whereas in the case of cervical cancer, a decrease in SL intensity was observed than that of normal subjects. It has been reported that, the biosynthesis of neopterin starts from the conversion of guanosine triphosphate to 7, 8-

Table 3 Cutoff value, AUC ± SE, Sensitivity and specificity of the ratio variables

Parameters	Cutoff value	AUC ± SE	Sensitivity [%]	Specificity [%]
I_{370}/I_{485} [V_1]	1.130	0.82±0.03	77.8	75.0
I_{397}/I_{485} [V_2]	1.082	0.90±0.02	83.3	83.0
I_{420}/I_{450} [V_3]	1.128	0.66±0.04	71.1	52.0
I_{420}/I_{485} [V_4]	0.728	0.94±0.02	86.7	80.0
I_{420}/I_{522} [V_5]	3.752	0.64±0.02	60.0	60.0
I_{440}/I_{450} [V_6]	0.959	0.78±0.04	80.0	61.2
I_{440}/I_{485} [V_7]	0.646	0.94±0.02	90.0	83.5
I_{440}/I_{522} [V_8]	3.152	0.66±0.04	62.2	61.2
I_{450}/I_{485} [V_9]	0.585	0.93±0.02	95.6	80.0
I_{485}/I_{522} [V_{10}]	4.016	0.10±0.03	30.0	11.8

dihydroneopterintriphosphate and subsequently to 5,6,7,8-tetrahydrobiopterin by pyruvoyl-tetrahydropterin synthase. However, due to the relative deficiency of pyruvoyl-tetrahydropterin synthase, human monocytes and macrophages on stimulation with interferon-γ produce and release increased amounts of neopterin in body fluids at the expense of biopterin derivatives [41]. It has also been reported that the distribution of pteridine and its derivatives may change when the monocytes and macrophages are activated under interferon-γ stimulus by cancer and may be the reason for neopterin elevation in urine [22]. The variations in the synchronous luminescence intensity of urine of normal subjects and cancer patients may be attributed to different pathologic conditions and their micro environment.

By considering the spectral differences between normal subjects and cancer patients, stepwise linear discriminant analysis and ROC analysis were carried out. Different ratio parameters were calculated and each wavelength used in these ratio parameters represents a specific characteristic spectral feature of one or more groups. For instance, I_{370} have been chosen to represent pteridine and I_{397}, I_{420}, I_{440}, and I_{450} have been chosen to represent riboflavin and pteridine groups. I_{485} and I_{522} represent the contribution of porphyrin. Out of 85 cancer subjects 76 are correctly classified and nine cases are misclassified as normal subjects yielding a sensitivity to detect cancer subject is 89.4 % and out of 90 normal subjects, 82 subjects are correctly discriminated and eight subjects are misclassified as cancer patients yielding a specificity of 91.1 % whereas (Table 2, Fig. 3). In this discriminant analysis, 90.30 % of the original grouped cases and 88.60 % of the cross-validated grouped cases were correctly classified. Also from the ROC analysis (Fig. 4, Table 3), it is found that the variables V_1, V_2, V_4, V_7 and V_9 have area under the curve closer to 1. From both the analysis, it is clear that the ratio variables calculated from the emission intensity of pteridines and flavins discriminates the cancer patients from that of normal subjects.

In conclusion, based on the spectral information and the results of statistical analysis, the SLS may be used in the discrimination of urine samples of cancer patients from that of normal subjects. For statistical analysis, the samples from different aetiology of cancer are grouped as cancer. However, further studies are required with more number of samples in each group to have discrimination between different stages and aetiologies of cancer with that of normal subjects.

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