

# The Factors Influencing Direct Spectral Fluorimetry of some Urine Metabolites

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Received July 22, 2010; Accepted November 11, 2010.

**Key words:** Autofluorescence of urine – Urine fluorophores – Tryptophan metabolites – Tyrosine metabolites

**Abstract:** Urine contains a variety of organic and inorganic chemicals including a number of natural fluorophores. Most of them are formed by tryptophan metabolites. But there are also metabolites of riboflavin, catecholamines and porphyrins. The alternation in the autofluorescence of urine and the alternation in the concentration of these substances are developed by both physiological and pathological changes such as disorder of body metabolism, dietary intake, age and etc. In this work we present fluorescent properties of chosen urine fluorophores – i.e. 5-hydroxyindole-3-acetic acid (5-HIAA), indoxyl sulphate (urine indican), serotonin (5-HT), vanillylmandelic (VMA) and homovanillic (HVA) acids typical for various diseases. Differences of fluorescent parameters of individual fluorophores measured *in vitro* in the water solutions and in natural environment of urine are significant and can lead to false results and conclusions. Therefore, we present the most common influence that can occur in urine (e.g. pH, ionic strength, proteins, and other fluorophores). The aim is to elaborate the exact “know-how” for direct complex fluorescent measurement in urine related to particular diagnoses.

*This study was carried out within projects VEGA 1/4233/07 and VEGA 1/0102/10.*

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## Introduction

Urine contains a variety of organic and inorganic chemicals including a number of natural fluorophores, most of which are tryptophan metabolites, but there are also metabolites of riboflavin, catecholamines and porphyrins. Due to these substances, normal urine has strong fluorescence. The alteration in the autofluorescence of urine, respectively the alternation in the concentration of these substances, is the result of both physiological and pathological changes which can occur due to a disorder of body metabolism, dietary intake, age and etc. (Anwer et al., 2009; Perinchery et al., 2010).

Fluorescent spectra of individual fluorophores differ from the spectra obtained from measurements of mixed fluorophores in the biological material (Dubayová et al., 2003). The main difference is in the known influencing of spectral parameters by the fluorophores interaction of the mixture. The behaviour of fluorophores associated with the characteristics of environment (differences of pH, ionic strength or protein concentration) is not really known.

Therefore, in this work we present results that specify the effect of defined environment of ionic strength, pH and protein on spectral properties of the individual fluorophores in urine. The complexity of urine may have an impact on the intensity of fluorescence, or may cause changes in the spectrum (shift of excitation and/or emission maximum) without any changes of individual fluorophore concentration. The above-mentioned effects have been studied in a model environment for the tryptophan metabolites – 5-hydroxyindole-3-acetic acid (5-HIAA), serotonin (5-HT), indoxyl sulphate (urine indican) and also vanillylmandelic (VMA) and homovanillic (HVA) acids which are the products of metabolism of tyrosine.

The studied fluorophores in urine are typical for various disorders. For example, carcinoid of: heart (Denney et al., 1998; Pandya et al., 2002), thyroid gland, gastrointestinal tract (van der Horst-Schrivers et al., 2007; Khan and Coleman, 2008) or appendicitis (Apak et al., 2005; Xu et al., 2007). Whipple disease, celiac disease and tropical spruce (de Jong et al., 2008) activate the increased concentration of 5-HIAA; on the contrary, Down's syndrome or Hartnup disease reduce the concentration of this fluorophore (Tasdemir et al., 2004). In addition to concentration of 5-HIAA, carcinoids also activate overproduction of serotonin (Lionetto et al., 2008). Increased levels of serotonin can be associated with the presence of mental disorders such as depression, schizophrenia, migraine and autism (Todoroki et al., 2006; de Jong et al., 2008). Pathologically increased concentration of indican in urine signals septic processes in the organism which may occur in intestinal obstruction, diarrhoea, Hartnup disease, gastric cancer and in lung abscesses (Allegri et al., 2003). The urinary excretion of VMA, HVA and 5-HIAA can be increased in the presence of neuroblastoma, ganglioneuroblastoma, ganglioneuroma and other tumours (Manickum, 2009). The former is characterized by defective catecholamine metabolism which results in high urinary levels of VMA and HVA. Later on, metabolic changes of tryptophan and the increased synthesis of serotonin, producing high 5-HIAA urinary concentrations can occur (Lionetto et al., 2008).

Therefore, our intention was to measure the effects of salts, pH and protein of chosen urine fluorophores so they could be used for mathematical analysis of complex spectra of this biological fluid.

### Material and Methods

Pure fluorophores – standards (Sigma-Aldrich) were dissolved in deionized water and further diluted by phosphate buffer with pH 5.7, 6.5, 7.3 and 8.0 to the concentration suitable for measurement. Concentrations of individual metabolites were not identical because of different fluorescence intensities (Table 1). The effect of ionic strength was modelled by the presence of NaCl (Lachema n.p. Brno) with concentrations 0.1, 0.2, 0.3 and 0.5 mol/l and in each pH environment. Proteins were modelled by the bovine albumin presence (Sigma-Aldrich) 20  $\mu\text{g/ml}$ , dissolved in deionized water. This measurement was carried out at pH=6.5 (near the physiological pH of urine). Spectral measurements were made by the fluorescent spectrophotometer Perkin-Elmer Model 3000 and Perkin-Elmer LS 55 in 10 mm quartz cell at laboratory temperature.

### Results and Discussion

Measurements of fluorescence properties of chosen urinary metabolites, typical for various disorders, showed relatively good spectral stability whilst considering changes of basic parameters of the environment (urine) – pH and ionic strength. The presence of increased concentration of proteins was revealed by the fluorescent intensity.

The value of pH is an important property of urine and physiologically it ranges from 5.5 to 6. The change of pH of environment has only a slight influence on the observed fluorophores. Increased pH value causes a little increase of fluorescence intensity of 5-HIAA and indoxyl sulphate, meanwhile intensities of VMA and HVA seem to be same (Figure 1). The exception is serotonin (5-HT), the dependence of which on pH environment has the opposite course, thus in the alkali environment its fluorescence decreases.

Ionic strength (concentration of salt) has almost none or only a minimal influence on spectral characteristics (Figure 2). Stronger effect, regardless of pH, was monitored in HVA and serotonin where, with increasing concentration of NaCl, a slight increase in fluorescence occurs.

**Table 1 – Spectral parameters of individual metabolites in the water environment**

Fluorophore	$\lambda_{\text{ex}}$ (nm)	$\lambda_{\text{em}}$ (nm)	$c$ (mol/dm <sup>3</sup> ) ( $\times 10^{-6}$ )	$c$ ( $\mu\text{g/ml}$ )
5-HIAA	276	341	0.201	0.075
Indoxyl sulphate	276	386	0.199	0.050
5-HT	277	341	0.549	0.117
HVA	277	318	12.810	2.330
VMA	279	315	5.045	1.000

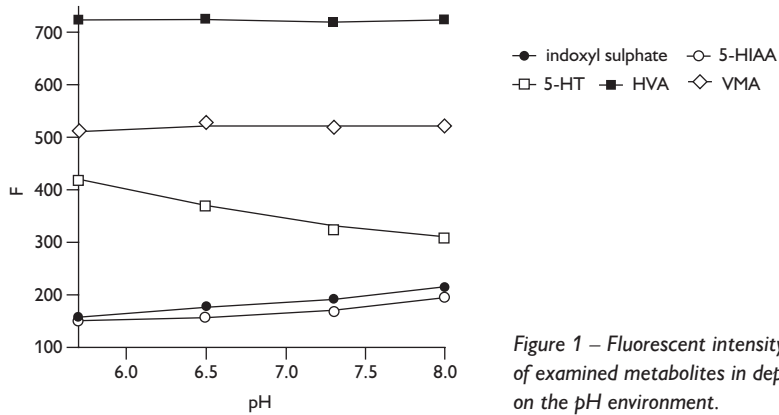


Figure 1 – Fluorescent intensity (F) of examined metabolites in dependence on the pH environment.

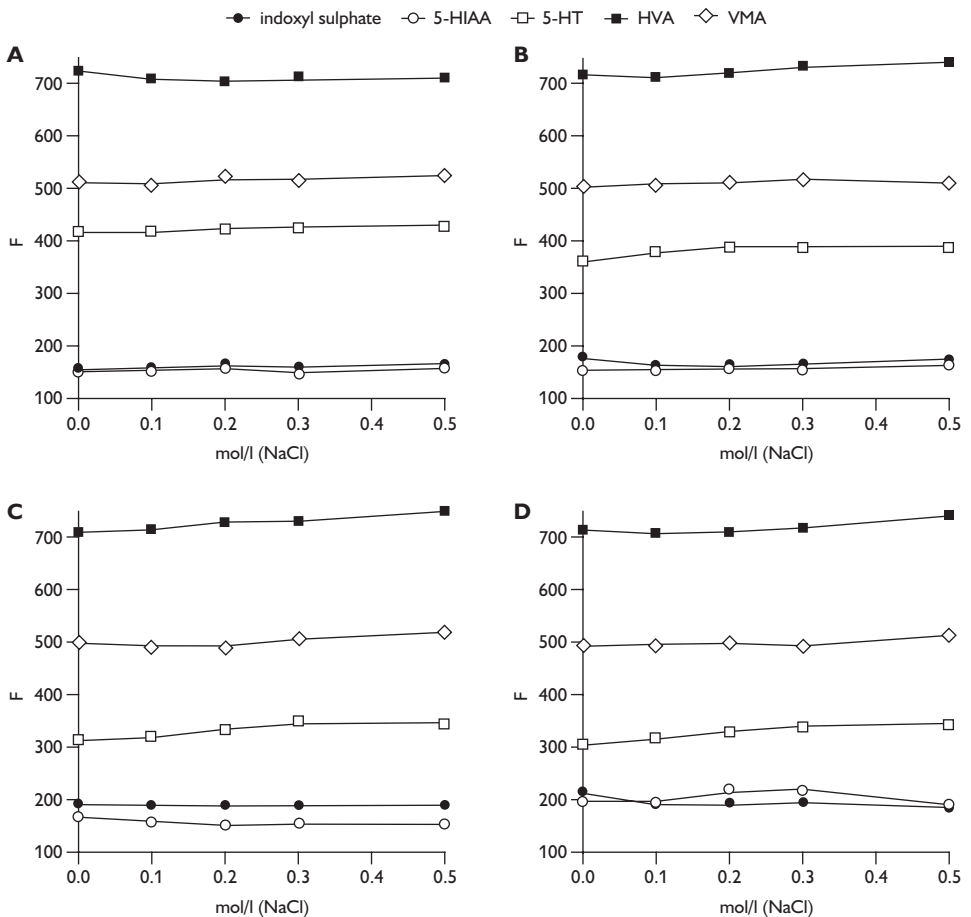


Figure 2 – Effect of increasing concentration of salt (NaCl) on intensity fluorescence (F) of chosen metabolites at: a) pH=5.7; b) pH=6.5; c) pH=7.3; d) pH=8.0. Changes of fluorescence are relatively small.

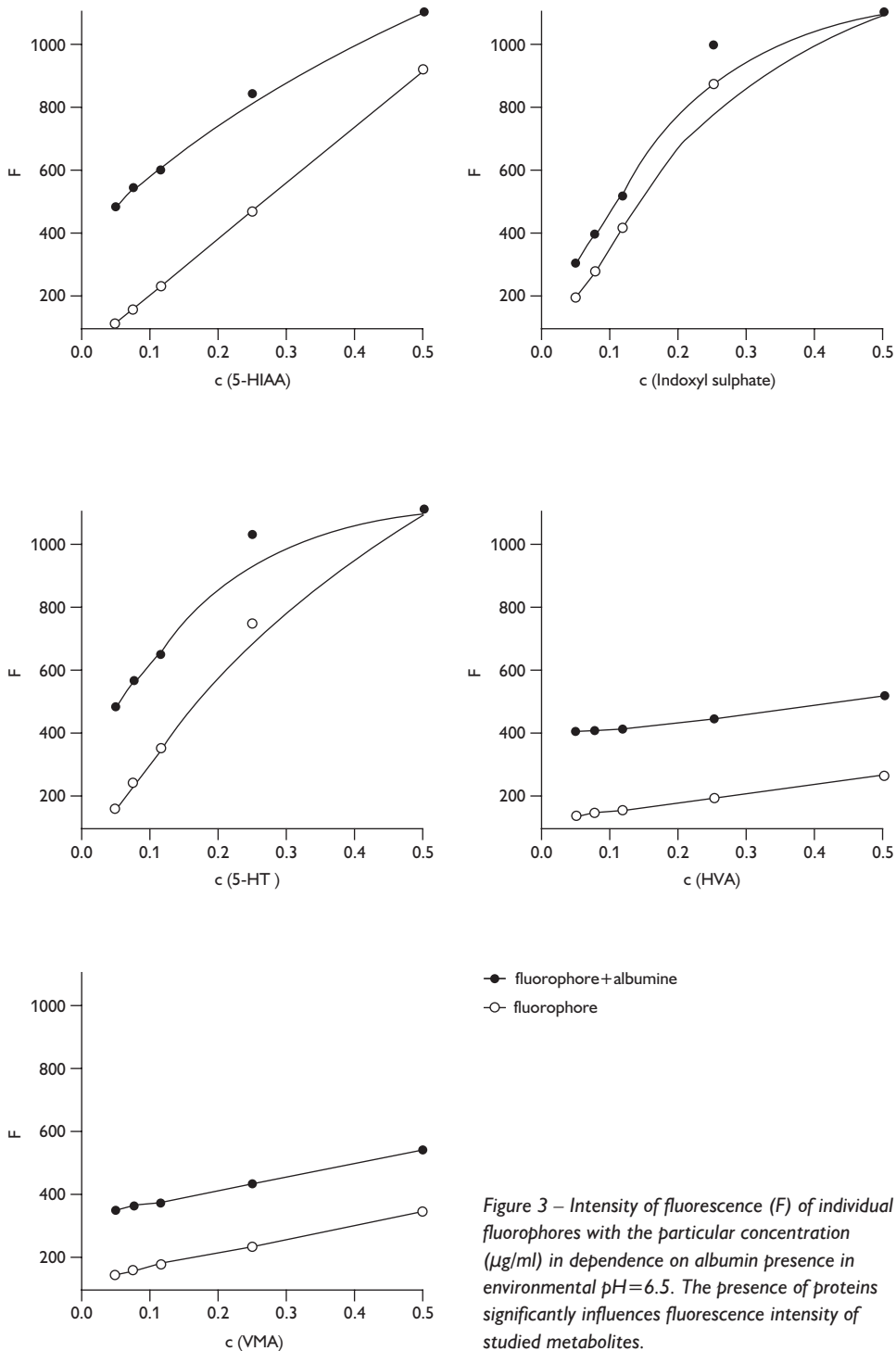


Figure 3 – Intensity of fluorescence (F) of individual fluorophores with the particular concentration ( $\mu\text{g/ml}$ ) in dependence on albumin presence in environmental  $\text{pH}=6.5$ . The presence of proteins significantly influences fluorescence intensity of studied metabolites.

Bovine serum albumin, a large globular protein (66,000 Da) (Tayeh et al., 2009), represents proteins whose increased presence in urine often occurs. By adding the albumin to each metabolite the increase of intensity of fluorescence occurred. It may be caused by nearby fluorescent characteristics of albumin and monitored substances (Figure 3). As this measurement was carried at wavelengths appertaining to particular fluorophore (Table 1), the increase of intensity of fluorescence after addition of albumin is not constant. The lowest increase of albumin fluorescence was observed in a mixture with indoxyl sulphate (Table 2).

Measurements showed that the fluorescent metabolites of tryptophan and tyrosine presented in urine when pH is changing and ionic strength of the environment are characterized by relatively high stability of their fluorescent parameters. The presence of protein is the only exception. This proves that the unwanted change of fluorescent spectra of these substances in mixed biological materials is caused mainly by the mutual interaction of fluorophores. Therefore, it is necessary to pay full attention on evaluating fluorescence of complex materials. The obtained facts enable us to simplify the mathematical solution of complex spectra (e.g. PCA, PARAFAC) by possible neglecting of common physiological changes of pH and ionic strength of urine at direct fluorimetry of these urinary metabolites. Consequently, this simplifies the process of mathematical decomposition of complex spectra.

## Conclusion

Changes of pH and ionic strength of the environment (urine) have not significant effect on the fluorescent parameters of tryptophan and tyrosine metabolites (intensity of fluorescence shifts of maxima in the spectra). On the contrary, the presence of protein increases the intensity of urine fluorescence also at specific exciting and emission wavelengths of individual fluorophores. It can cause false positive results, and therefore, the concentration of protein in urine should always be tested.

Whilst measuring the autofluorescence of natural urine samples of patients it is necessary to focus primarily on the mutual influence of fluorophores (energy transfer, quenching, summary of several peaks in the spectrum, etc.) which mostly

**Table 2 – Intensity of fluorescence for individual fluorophores and albumin at pH=6.5; c (fluorophore) = 0.25 µg/ml and c (albumin) = 20 µg/ml**

Fluorophore	Intensity of		
	fluorophore fluorescence	albumin fluorescence	both fluorescences
5-HIAA	468	598	841
Indoxyl sulphate	874	149	999
5-HT	750	532	1034
HVA	104	347	378
VMA	151	332	371

affects the common spectrum of mixed materials. Physiological variations of pH and ionic strength influence the spectrum minimally and in the verified cases may be neglected.

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