



## BIOGENIC AMINES CONTENT IN THE SERUM OF PATIENTS WITH *NON-HODGKIN'S* LYMPHOMA

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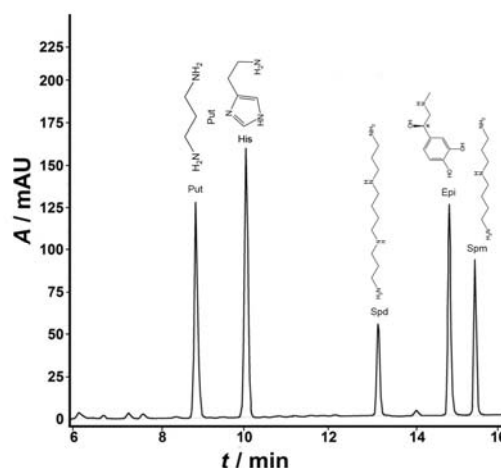
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The biosynthetic pathway of biogenic amines is very active during the growth of many tumour cells. Non-Hodgkin lymphoma represents a very heterogeneous group of malignancies. The concentration level of some biogenic amines (putrescine, histamine, spermidine, epinephrine, and spermine) was investigated to elucidate whether they could be observed as markers for the patients with *non-Hodgkin's* lymphoma. In this study, the method of acidic extraction of five biogenic amines from human serum, dansyl chloride pre-column derivatization, and LC/DAD analysis were used to determine the content of biogenic amines in biological fluids. The results indicate that statistically significant differences exist in putrescine, spermidine and histamine contents in *non-Hodgkin's* patients versus healthy controls. The concentrations of putrescine, spermidine and epinephrine were elevated, and histamine was lower compared to controls. Based on their content, serum biogenic amines could be considered as potential tumour markers.



### INTRODUCTION

Biogenic amines play various important roles in many biological systems.<sup>1-6</sup> The polyamines, as polycations, could stabilize their structure by interacting with biomolecules that contain negatively charged moieties such as phospholipids, proteins, DNA, and RNA.<sup>4</sup> Polyamines putrescine (Put), spermidine (Spd), and spermine (Spm) are very important for cell growth and their concentrations in cells increase rapidly during the period of cell proliferation, while catecholamines epinephrine (Epi) and norepinephrine (Nor) play a crucial role in the nervous system,<sup>7-9</sup> and *via*

adrenergic and dopaminergic receptors are included in the regulation of the response to stress, sleep, psychomotor activity, memory, and emotional processes.<sup>10-13</sup> Furthermore, it is proven that histamine (His) is the main mediator of immediate hypersensitivity and acute inflammatory responses of the cells, and also it can affect many functions of cells involved in the regulation of immune response and hematopoiesis including T and B lymphocytes as well as endothelial cells and dendritic cells.<sup>14</sup> The biosynthetic pathway of biogenic amines is very active during the growth of many tumor cells especially when metastasized.<sup>15-17</sup> The interest for biogenic amine contents in cancer

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patients compared to healthy subjects<sup>18,19</sup> rose since significant differences have been found in the polyamines content in the urine of patients with advanced gastric carcinoma, acute myelocytic leukaemia, and ovarian cancer in comparison to healthy subjects.<sup>20</sup> Nowadays there is plenty of scientific articles regarding biogenic amine concentrations in solid tumours, mainly used for diagnosis of neuroendocrine tumours such as pheochromocytoma and carcinoids,<sup>21,22</sup> but only a few regarding Non-Hodgkin's lymphoma and serum analyses.<sup>23,24</sup>

Non-Hodgkin's lymphoma represents a very heterogeneous group of malignancies with widely varying characteristics and clinical outcome.<sup>25–27</sup> It is generally accepted that the determination of biogenic amines in biological fluids can provide useful information in the assessment of cancer chemotherapy, as well as the detection of remission and recurrence.<sup>28</sup> Contents of the body polyamine can be most directly reflected by the biogenic amines in serum.<sup>29</sup> Since biogenic amines have a low molecular weight, their efficient detection depends on the proper sample preparation which is necessary before the analytical instrumentation purifies the analytes.<sup>30–32</sup> Chromatographic techniques are most commonly used for the determination of biogenic amines, but as some of them do not absorb in the UV/Vis field of the spectrum, it is necessary to do their derivatization.<sup>33</sup> Recently, we investigated the serum concentrations of specific biogenic amines in three immune-mediated diseases: *diabetes mellitus*, chronic *urticaria* and Hashimoto's thyroiditis, by using LC/DAD technique, to elucidate are there any changes in their contents in patients with these diseases compared to healthy controls.<sup>34</sup> Inspired by our previous study, the aim of this work was to investigate the concentrations of biogenic amines

(Put, Spd, His, Epi, and Spm, Figure 1), as dansylated derivatives, in the serum of NHL patients. As liquid chromatography is increasingly used in clinical biochemistry, especially in the field of low molecular weight biomarkers, our aim was to investigate whether a simple and relatively easy and fast biochemical method with pre-column derivatization, gradient elution, and ultraviolet detection could be used as diagnostic tool for diagnosis of non-Hodgkin's lymphoma.

## RESULTS

The most promising method for absolute recovery of low molecular weight substances from complicated systems is the acidic precipitation/extraction procedure.<sup>35</sup> Optimal conditions of experiments were found using different concentrations of HClO<sub>4</sub> as a precipitating agent (from 0.2–2.0 M), incubation time (5–40 min) and temperature (0–50 °C). The sharpest peaks of biogenic amines in chromatograms were found with 0.4 M HClO<sub>4</sub> and room temperature with the time of incubation of 30 minutes. The findings are in the complete agreement with the work of Mao *et al.*<sup>36</sup>

### Linearity

Slope calibration, intercept, and the correlation coefficient were obtained using least-square regression. Calibration curves for Put, His, Spd, Epi, and Spm were created after plotting obtained peak area values against calculated concentrations with excellent correlation coefficients (1.0; 1.0; 0.9999; 0.9999; 0.9998 respectively, Figure 2). Linearity was obtained for the range of 8–2.5×10<sup>4</sup> ng/L.

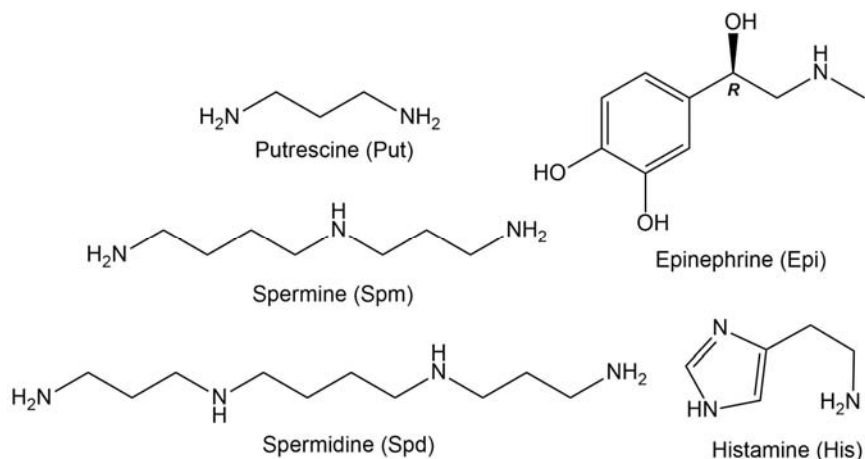


Fig. 1 – Structural formulas of investigated biogenic amines.

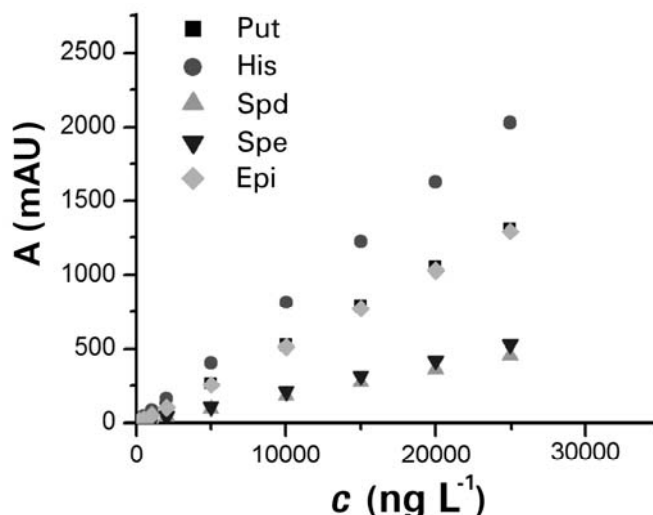


Fig. 2 – Calibration curves for Put, His, Spd, Epi and Spe.

Table 1

The concentration of tested biogenic amines (in ng/L  $\pm$  SD) in healthy subjects and *non-Hodgkin's* lymphoma (NHL) patients

| Analyte (ng/L)    | Putrescine                  | Histamine                 | Spermidine                 | Epinephrine                 | Spermine |
|-------------------|-----------------------------|---------------------------|----------------------------|-----------------------------|----------|
| Controls (n = 20) | 1954 $\pm$ 280              | 279 $\pm$ 84              | 893 $\pm$ 136              | 354 $\pm$ 60                | < 200*   |
| NHL (n = 20)      | 2432 $\pm$ 312 <sup>+</sup> | 185 $\pm$ 89 <sup>+</sup> | 931 $\pm$ 120 <sup>+</sup> | 404 $\pm$ 113 <sup>++</sup> | < 200*   |

\*below LOD

<sup>+</sup> P<0.05 when compared with healthy controls (Student t-test)

<sup>++</sup> P>0.05 when compared with healthy controls (Student t-test)

### Accuracy and precision

We evaluated the repeatability by examining quality control samples six times per day, at three different concentrations, and intermediate precision by analysing the same samples daily for two days. Precision was taken as the relative standard deviation (RSD) of concentrations which were obtained from corresponding calibration curves. The obtained results meet the accuracy in the range from 96.01 – 99.80%, while the precision RSD values have been  $\leq$  7.25%.

### Limits of quantitation (LOQ) and detection (LOD)

The concentration was considered as LOQ when the signal/noise ratio was at least 10:1. The LOD was identified as a signal/noise ratio of 3:1.<sup>34</sup>

### Recovery experiment

Serum samples with known concentrations (putrescine, histamine, spermidine, epinephrine<sup>37</sup> and spermine (1000, 2000, 3000 ng/L)) were spiked and

detected five times, and the rate of recovery of each standard has been determined. The average recovery rate is 99% for Put, 100.1% for His, 101% for Spd, 83% for Epi, and 100% for Spe.

### The content of biogenic amines in serum of controls and patients

Under specified experimental conditions all standard amine peaks appeared within 20 min, without overlapping. The obtained retention times were: 8.9, 10.1, 13.1, 14.7, and 15.4 minutes for Put, His, Spd, Epi, and Spe, respectively. The content of investigated biogenic amines in healthy subjects and NHL patients are showed in Table 1.

## DISCUSSION

Herein, the contents of putrescine, histamine, spermidine, epinephrine, and spermine in the serum of healthy controls and patients with Non-Hodgkin's lymphoma (NHL) were investigated with the aim to determine whether these compounds can be used as markers for the

malignant process. It is noticed that concentrations of some particular polyamines are very different in some patients with NHL, compared to healthy subjects. Differences, observed by parameters of descriptive statistics, were evaluated by the two tailed Student's *t* test. The statistically significant difference exists in putrescine concentrations in both groups of subjects and is higher in those with NHL compared to healthy once ( $P < 0.0302$ ). Spermidine contents are also statistically significantly higher in tumour group than in healthy subjects ( $P < 0.0331$ ), but histamine concentrations are found to be statistically lower in patients with NHL ( $P < 0.0017$ ) in comparison with controls. Epinephrine contents in patients are also elevated, however not statistically significant than in healthy controls ( $P < 0.1432$ ). The rationale behind this observation may lay presumably in fact that adrenal glands in patients were not impacted by NHL. Spermine in serum samples in both controls and patients was under the limit of detection. Findings from this study supported general expectations. Tissues, which are growing rapidly, especially malignant tissues, expel the highest amounts of biogenic amines, namely putrescine and spermidine which is in accordance with the work of Thyss *et al.*<sup>35</sup> The results of the study indicate that biogenic amines are possible markers of malignant diseases, so their detailed investigation is of great importance. It would be interesting to investigate the correlation between biogenic amine contents depending on the acuteness and the extent of the disease. Presumably, the usage of chemotherapy and medications lowers the concentrations of biogenic amines to the normal range. Non-Hodgkin's lymphoma is a varied group of lymphoproliferative malignancies. It shows different patterns of behaviour and responses to treatment. It is widely recognized that the microenvironment has a great influence on neoplastic cells. Infiltrate of inflammatory cells, specifically macrophages, lymphocytes, mast cells, and neutrophils, are surrounding the Lymphoma cells.<sup>37</sup> Mast cells are a major source of histamine, which can affect tumour growth, while suppressing the immune system. Having lower contents of histamine than in healthy controls could mean that immune system is already being suppressed because of the disease, yet released histamine acts antiproliferative against NHL expander. In addition to immune system involvement in tumour suppression is the epinephrine contents examined herein. Higher values of serum epinephrine in patients, compared

to controls are supported by adrenal hypothesis,<sup>38</sup> which proposes that elevation in plasmatic adrenal hormones provokes elimination of malignant cells. Recently, it was described that human body might produce greater quantities of catecholamines as a natural protection against (immune-related) disorders, simultaneously suppressing the histamine contents.<sup>34</sup> Results reported in this study for epinephrine also indicate that patients do not suffer from adrenal NHL, which is very rare and accounts less than 1% of all NHL cases. Thus, this may be an excellent basis for forthcoming examination of serum samples of various types of NHL, and even their comparison with Hodgkin's lymphoma. Siu *et al.* showed that administration of medication induces a consistent suppression and up to 70% reduction in spermine.<sup>39</sup> However, concentrations of spermine in this study were below limits of detection, thus no difference between patients and control group were observed. Data obtained in this work suggest that three from five investigated biogenic amine contents in serum indicate the presence of malignant process and could be observed as markers for NHL. Moreover, recently for immune-mediated diseases (diabetes mellitus, chronic urticaria, Hoshimoto's thyroiditis) was shown that similar ratio of biogenic contents in patients compared to healthy subjects was detected.<sup>34</sup>

## EXPERIMENTAL

Chemicals used in experiments are of analytical HPLC grade. Standards of biogenic amines are in the form of hydrochloride salts of the highest purity (Acros Organics, Beel, Belgium).

### Serum preparation

The whole blood was drawn from the vein in a Vacutainer Venous Blood Collection tube. No anticoagulant was used. The whole blood was allowed to clot for 30 minutes by leaving it undisturbed at room temperature. By centrifuging at  $2,000 \times g$  for 10 minutes in a refrigerated centrifuge, the clot was removed. The resulting supernatant, designated as serum, has been immediately transferred into a clean polypropylene tube using a Pasteur pipette and stored until analysis at  $-20^{\circ}\text{C}$ .

### Characteristics of patients

In this study 20 healthy volunteers and 20 patients with diagnosed Non-Hodgkin's lymphoma from Institute of Oncology and Radiology of Serbia were included. Cancer stadium and category were not taken as significant for the study. Healthy controls were randomly selected and did not have any reported diagnosis. All participants in this study provided a written consent before starting the experimental examination.

### Sample preparation

750  $\mu\text{L}$  of 0.4 M  $\text{HClO}_4$  was added to a 500  $\mu\text{L}$  of serum sample in micro test tubes, vortexed for 1 min, and centrifuged at 13000 rpm for 10 min. The supernatant was transferred into another test tube, and afterwards 67.5  $\mu\text{L}$  of 2.0 M NaOH was added and vortexed for 1 min. To the test tube was added 150  $\mu\text{L}$  of a saturated solution of sodium bicarbonate, and pH was adjusted to 8.0. Dansyl chloride (1000  $\mu\text{L}$  of 10 g/L) was added to prepared samples and mixture was vortexed for 1 min, and then left in water bath, heated at 40  $^\circ\text{C}$  for 45 minutes. Lastly, for removal of the excess of derivatization reagent, 50  $\mu\text{L}$  of 25% ammonium hydroxide was added, incubated for 30 min, at room temperature, avoiding light. All samples have been filtrated across 0.45  $\mu\text{m}$  Econofilters before injection.<sup>36</sup>

### Method validation

Standard solutions of Put, His, Spd, Epi and Spe have been used for external calibration procedure in the aim of obtaining data for quantitative analysis. The method validation has been achieved by the examination of linearity, accuracy and precision, limit of quantitation (LOQ) and detection (LOD) as well as recovery. The linearity of the method has been performed for biogenic amine standards by injecting 50  $\mu\text{L}$  volumes of each standard solution into LC/DAD system. The concentration range for amine standard solutions was among 5 ng/L and  $3 \times 10^4$  ng/L. The accuracy of the used analytic method has been determined as the percentage relative error. The limit of quantitation and detection of investigated compounds were confirmed by injecting gradually low concentration of the standard solution under the experimental conditions. The recovery has been accomplished by spiking techniques, using the real serum sample from a healthy control which has been examined in the same way as other samples.

### Statistical analysis

Statistical analysis of experimental data was performed using by two-tailed Student's *t*-test.

## CONCLUSION

In this study, the content of putrescine, histamine, spermidine, epinephrine, and spermine was investigated to elucidate whether they could be observed as markers for malignant diseases. Most of the data in the literature relate to polyamine concentrations in the urine and relatively few in the serum. As malignant diseases are in expansion, the aim of our work was to investigate biogenic amine contents, as dansylated derivatives, in the serum of non-Hodgkin's patients, using LC/DAD technique, so we could apply it in clinical practice. Statistically significant differences are found in putrescine, spermidine, histamine and epinephrine concentrations in non-Hodgkin's patients versus healthy controls, which was in accordance with our expectations. The

content of putrescine, spermidine and epinephrine were elevated, while histamine was lower compared to controls. It may be summarized that whenever the immune-related process occurs the biogenic amines could be used as markers but they might not be sensitive in terms of stratification of diseases-malignancies. Since there is a fine line between immune suppression and tumours, biogenic amines appear to be significant in understanding these disorders. Possible nutrition behaviours effect on the content of biogenic amines in blood serum could also be the topic of some future analyses.

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