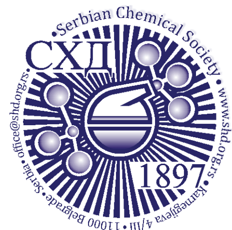

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LC/DAD determination of biogenic amines in serum of patients with diabetes mellitus, chronic urticaria or Hashimoto's thyroiditis

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Abstract. Biogenic amines are integral part of nearly every cell. In the present study, the method of acidic extraction of histamine (His), of polyamines putrescine (Put), spermidine (Spd) and catecholamines epinephrine (Epi) and norepinephrine (NE) from human serum, precolumn derivatization with dansyl chloride, and LC/DAD analysis of the biogenic amines was used with the aim of monitoring differences of their levels in patients with diabetes mellitus, chronic urticaria, and Hashimoto's thyroiditis, compared to healthy subjects, and to observe them as possible markers for immune mediated diseases. The retention times were used for the determination of serum biogenic amines. Statistically significant differences were found in the levels of putrescine and histamine in diabetes mellitus patients, of the levels of putrescine, histamine, spermidine and epinephrine in chronic urticaria patients and of the levels of putrescine and spermidine levels in Hashimoto's thyroiditis patients, compared to those of healthy controls. Norepinephrine was found only in the serum of patients with chronic urticaria. The values of recovery, evaluated in controls, varied between 85.7 and 106.7 %. The statistically significant changes in putrescine, histamine, spermidine and epinephrine levels in patients compared

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with those in healthy people reflects the existence of biochemical disturbances in the mentioned immune-mediated diseases.

Keywords: biogenic amines; serum; immune-mediated diseases; markers, LC/DAD.

INTRODUCTION

Polyamines and catecholamines are members of a broad group of the so-called biogenic amines.[1] Since the first description (by Antoni van Leeuwenhoek in 1678) of crystals that were much later identified as polyamine salts, these molecules have been the subject of intense research efforts, which have shed light on several biological and pathological processes, for example the repair of the extracellular matrix, cell adhesion, synthesis of nucleic acids, and involvement in cellular growth and proliferation. These molecules are also related with several types of cancer, such as breast and colon cancer, adenocarcinoma, non-Hodgkin's lymphoma *etc.*[2,3] Histamine and the polyamines putrescine and spermidine form a group of naturally occurring compounds that exert a large number of biological effects, but the mode of action of these biogenic amines at the molecular level is largely unknown.[4–7]

Catecholamines – epinephrine and norepinephrine containing an amino function on a side chain together with the catechol function (*i.e.* hydroxyl groups at the 3- and 4-positions on a benzene ring) play an important role in the nervous, cardiovascular and endocrine systems. Catecholamines act *via* adrenergic receptors, and are involved in the regulation of the response to stress, psychomotor activity, emotional processes, learning, sleep, and memory.[1,8–12] Norepinephrine is also useful in the diagnosis of pheochromocytoma.[13]

Polyamines as polycations are essentially involved in cell growth and differentiation.[14,15] They influence the transcriptional and translational stages of protein synthesis, stabilize membranes, modulate neurophysiological functions and may act as intracellular messengers.[2,4] Hence, the majority of the polyamines will be sequestrated in some way and it is probable that only the "free" polyamine pool is physiologically active.[16] Since intracellular polyamine concentrations are much greater than extracellular, a small amount of cell lysis can markedly alter their obtained values.[17]

Polyamines appear to have a role in immune-mediated diseases[18] and have effect on DNA/RNA structure, chromatin condensation, translation, protein synthesis and mammalian cell growth.[19] In humans, the inhibitor of polyamine biosynthesis, α-difluoromethylornithine (DFMO), with an inhibitor of cyclooxygenase 2 (COX-2) proved to be effective in prevention of the recurrence of colon cancer and adenomas, associated with the development of colon cancer.[19] Acetylated spermine is increased in urine in different types of human cancers.[19] In colon cancer, the sensitivity of the detection of diacetylspermine (DiAcSpm) in

urine is much higher and in breast cancer noticeably higher than the sensitivity of regularly used diagnostic serum biomarkers.[20]

Quantification of biogenic amines in biological fluids has an important diagnostic implication in medicine,[21] but direct determination of biogenic amines in biological fluids is difficult because they are small aliphatic or aromatic molecules that do not exhibit any structural features that would allow sensitive and selective detection.[22] Chromatographic methods are generally used for the determination of biogenic amines, but, since only few of them absorb in the UV–visible region, for their simultaneous determination, a pretreatment step of derivatization is generally performed.[23] Among a wide panel of analytical methods developed for the quantification of biogenic amines, HPLC separation after derivatization with dansyl chloride remains the most commonly used method.[24] In this study, pre-column derivatization, gradient elution, and ultraviolet detection with a DAD detector were used.

First, the serum levels of specific biogenic amines in three immune-mediated diseases, *i.e.*, diabetes mellitus, chronic urticaria and Hashimoto's thyroiditis, were investigated to elucidate whether there were any changes in their levels in mentioned diseases compared to healthy people. In order to determine the concentrations of serum biogenic amines in these diseases and to assess the differences among their levels,[19,24,25] the method of Ben-Gigirey *et al*. modified by Mao *et al.* was used because equipment similar to theirs was available, thereby providing reproducible results.

MATERIALS AND METHODS

Reagents and chemicals

All chemicals were of analytical HPLC grade. All biogenic amine standards were purchased as hydrochloride salts of the highest available purity. The standards of 1,4-diaminobutane dihydrochloride (putrescine), 99+ %, spermidine trihydrochloride, 99+ %, histamine dihydrochloride, 99 %, and L-(–)-epinephrine, 99 %, were purchased from Acros Organics (Beel, Belgium). (–)-Norepinephrine, 99.5 %, was purchased from Sigma (St Louise, MO, USA). The derivatization reagent, dansyl chloride, 98%, was procured from Acros Organics. Perchloric acid, sodium hydroxide, sodium bicarbonate, 25 % ammonium hydroxide, acetone, acetonitrile, and ammonium formate were from Merck (Darmstadt, Germany). Ultra-pure HPLC grade water was prepared by passing doubly deionized water through a Milli-Q system.

Individual standard solutions of the biogenic amines were made by dissolving an exactly weighed amount of the required standard in ultra-pure water. After dissolution, each standard was diluted to 1 mg L^{-1} with ultra-pure water and stored as a stock solution at 4 °C in the dark. Working standard dilutions were freshly prepared by diluting the stock standard solutions with ultra-pure water for each assay. A solution of dansyl chloride (the derivatization reagent) was made in acetone, diluted to 10 mg L^{-1} and then stored at 4 °C in the dark.

Serum preparation

Whole blood was collected after venipuncture in covered red top test tubes – Vacutainers (BD – Becton Dickinson), without any anticoagulant. After collection of whole blood, the blood was left undisturbed to clot for 15–30 min at room temperature. The clot was removed by centrifuging at 2000*g* for 10 min. The resulting supernatant was designated serum. Following centrifugation, the liquid component (serum) was immediately transferred into a clean polypropylene tube using a Pasteur pipette. The serum samples were apportioned into 0.5 mL aliquots and stored at –20 °C until analysis.

Patients' characteristics

The present study included patients from the University Clinical Centre "Zvezdara" Hospital (Belgrade, Serbia) or the Clinical Centre of Serbia (Belgrade, Serbia) and 20 healthy controls. Three medical conditions/diseases – diabetes mellitus (Type I and Type II), chronic urticaria and Hashimoto's thyroiditis, which were newly diagnosed, were investigated in the serum. 12 of 20 diabetes mellitus patients were men, and 8 were women (age range 53–64, median age 59); 6 of 20 chronic urticaria patients were men, and 14 were women (age range 24–77, median age 48), and 8 Hashimoto's thyroiditis patients were women (age range 33–75, median age 48). The chosen patients had no additional disorders beside the investigated one. Healthy controls, of which 11 women and 9 men (age range 27–62, median age 36), were randomly chosen and did not have any reported diagnosis. All patients and controls provided written informed consent before entry into the study.

Apparatus

The HPLC system comprises a liquid chromatograph Agilent 1200 Series (Agilent Technologies, Santa Clara, CA, USA), with a degasser, a binary pump, an autosampler, a column compartment equipped with a Zorbax Eclipse Plus C18 column (150 mm×4.6 mm; 5 μm), and a diode-array detector (DAD). A personal computer system running ChemStation Software (Agilent Technologies, Santa Clara, CA, USA) was used for data collection and processing.

Chromatographic conditions

A mixture of solvents A (water containing 5 mM ammonium formate) and B (acetonitrile) in a gradient mode of elution was used as the mobile phase. The gradient program was selected as follows: 55–65 % B, 0–6 min; 65–100 % B, 6–15 min; 100 % B, 15–19 min; 100––65 % B, 19–20 min; 65 % B, 20–24 min. The mobile phase solutions were pumped at a flow rate of 1.0 mL min^{-1}. Signals were accumulated in the wavelength range 190–450 nm using a DAD, and the chromatograms were recorded at 254 nm. The injection volume was 50 μL. The temperature of the column was kept at 40 °C.

Standard treatment

The stock standard solutions of each biogenic amine were diluted with ultra pure water to the desired concentrations. Each standard (500 μL) was worked up as described above for the serum samples. The qualitative analysis was realized using the retention times. The quantitative analysis was performed using the corresponding external calibration. The calibration curves were obtained by plotting the biogenic amines peak area values against the respective concentrations of biogenic amines standards.

Sample preparation

The method proposed by Mao *et al*. was used.[25] To 500 μL of serum sample in a micro test tube, 750 μL of 0.4 M perchloric acid was added, then vortexed for 1 min, and centrifuged

at 13000 rpm for 10 min. The supernatant was aspirated into another test tube followed by the addition of 67.5 μL of 2.0 M NaOH, vortexed for 1 min and then 150 μL of a saturated solution of sodium bicarbonate was added into the test tube to adjust the pH to 8.0–8.5. To a prepared sample 1000 μL of 10 g L^{-1} dansyl chloride was added, and the mixture was vortexed for 1 min, and left to react in a closed water bath at 40 °C for 45 min. Finally, 50 μL of 25 % ammonium hydroxide was added to remove the excess derivatization reagent, and incubated for 30 min, at room temperature, in the dark. Before injection, all samples were filtrated through 0.40 μm Econofilters.

Validation of the method

All quantitative analysis were performed by the external calibration procedure using standard solutions of putrescine, histamine, spermidine, epinephrine and norepinephrine. The validation of the method was accomplished by the test of linearity, precision and accuracy, limit of detection (*LOD*), limit of quantification (*LOQ*) and recovery. The linearity of the method was tested for the biogenic amine standards by injecting 50 μL of each amine standard solution within the concentration range of $5–50\times10^3$ ng L^{-1} into the LC/DAD system. The accuracy of this analytic method was assessed as the percentage relative error. The *LOD* and *LOQ* values for the biogenic amines were determined by injecting progressively lower concentration of the standard solution under the chromatographic conditions. The recovery study was performed using a real serum sample from a healthy control, by spiking techniques. This control serum sample was treated in the same way as were the other samples. The recoveries were checked as three different concentration levels (low, medium and high).

Statistical analysis

Descriptive statistics and the Kruskal–Wallis test together with the multiple-comparison Z-value test were used for statistical analysis of the experimental data employing a demo version of the Number Cruncher Statistical Systems software (Kaysville, UT, USA). The linear relationship between peak areas and concentrations was calculated by linear regression.

RESULTS AND DISCUSSION

Optimal conditions of sample processing

The acidic precipitation/extraction procedure is the most promising method for complete recovery of low molecular weight compounds from complex matrices.[1] A solution of 0.4 M $HClO_4$ gave slightly sharper peaks for the biogenic amines in the chromatograms, which is in agreement with the work of Mao *et al.*[25]

Linearity

Least-square regression was used for the determination of the slope, intercept, and the correlation coefficient of the calibration curves, constructed by plotting the peak area values against the respective concentrations. Linearity was obtained for the range of $80–5\times10^4$ ng L^{-1} (Fig. S-1 of the Supplementary material to this paper) and the correlation coefficients were excellent (Table S-I of the Supplementary material).

Precision and accuracy

The repeatability was evaluated by analyzing the quality control samples, at three different concentrations, six times per day. The intermediate precision was evaluated by analyzing the same samples once daily for two days. The relative standard deviation (*RSD*) of the concentrations obtained from the corresponding calibration curves was taken as the precision. The results, which are summarized in Table S-II of the Supplementary material, meet the requirements of accuracy within 95.12–99.75 %, and the precision *RSD* values were ≤ 8.46 %.

Limits of detection (LOD) *and quantification* (LOQ)

The lowest concentrations assayed where the signal/noise ratio was at least 10:1 was regarded as the *LOQ*. The *LOD* was defined as the concentration when the signal/noise ratio was 3:1.

The determined *LOD* values were 40 ng L^{-1} for putrescine, 30 ng L^{-1} for histamine, 250 ng L^{-1} for spermidine, 90 ng L^{-1} for epinephrine and 596 ng L^{-1} for norepinephrine.

The determined *LOQ* values were 150 ng L^{-1} for putrescine, 90 ng L^{-1} for histamine, 820 ng L^{-1} for spermidine, 300 ng L^{-1} for epinephrine and 1980 ng L^{-1} for norepinephrine.

Recovery experiment

Serum samples with known concentrations of biogenic amines, were spiked with concentrations of putrescine and histamine (1000, 1500 and 2000 ng L^{-1}), spermidine (2000, 4000 and 6000 ng L^{-1}), epinephrine (350, 700 and 1000 ng L^{-1}), and norepinephrine (1500, 3000 and 5000 ng L^{-1}). The serum concentration of each biogenic amine was determined five times, and the recovery rate of each standard was calculated. The chromatogram of a spiked serum sample is presented in Fig. S-2 of the Supplementary material. The average recovery rate ranged from 85.7 to 106.7 %.

The obtained data (Table S-III of the Supplementary material) showed good recovery values for all the investigated biogenic amines.

Detection of the levels of the biogenic amine in the serum of the controls and patients

A chromatogram of a mixture of the standard biogenic amines is shown in Fig. 1. All the standard amine peaks, under the given experimental conditions, appeared within 20 min, without overlapping. The retention times were 8.9 min for putrescine, 10.1 min for histamine, 13.1 min for spermidine, 13.9 min for norepinephrine and 14.7 min for epinephrine.

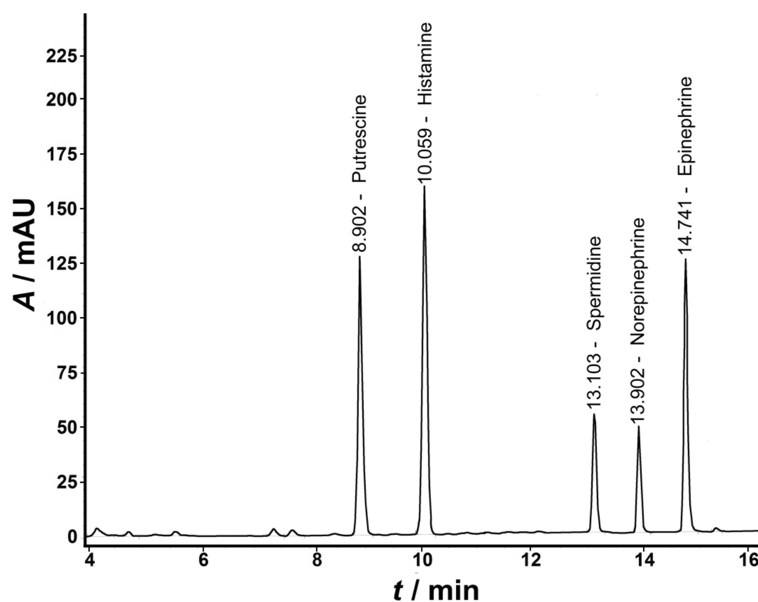

Fig. 1. A chromatogram of a mixture of the investigated serum biogenic amines.

The peaks of the investigated biogenic amines in the serum samples could also be separated within 20 min. Occasionally, the recording was spoiled by residues of the protein precipitate passing through the filter. This was resolved by additional centrifugation of the sample. Representative chromatograms of biogenic amines in the sera of the control subjects and patients with the investigated diseases are shown in Fig. 2.

The concentration levels for the five serum biogenic amines, determined in controls, and in patients are given in Table I. Differences, observed by parameters of descriptive statistics, were evaluated by the Kruskal–Wallis test followed by the multiple-comparison *Z*-value test. The putrescine concentrations in the sera of patients with diabetes mellitus, chronic urticaria and Hashimoto's thyroidits patients were lower compared to those in the sera of the healthy controls ($P < 0.0001$ and *Z*: C(D,U,H). The histamine levels were also statistically significant different between healthy controls and patients with diabetes mellitus or chronic urticaria ($P < 0.0001$ and *Z*: D(C,H,U)), while patients with diabetes had higher histamine levels than healthy subjects ($P < 0.0001$). Unexpectedly, in serum of chronic urticaria patients, the histamine levels were lower than in healthy controls ($P < 0.0001$). Eight patients with Hashimoto's thyroiditis had detectable histamine in their serum, of which four values were the same as in the sera of the healthy controls, while the other four were lower. The histamine levels in the sera of patients with Hashimoto's thyroiditis were also lower compared to those in the sera of patients with diabetes mellitus ($P < 0.0001$). Spermidine levels in the serum

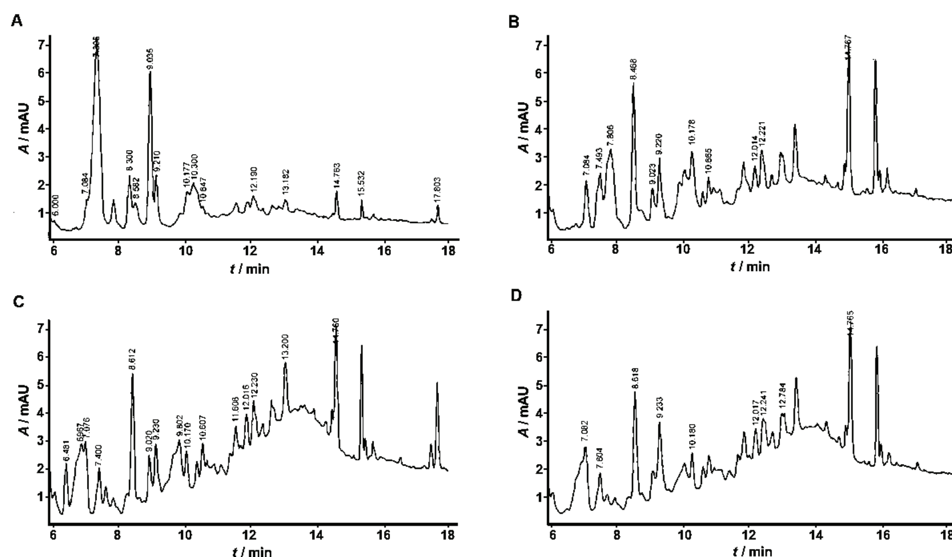

Fig. 2. Representative chromatograms of serum biogenic amines in: A) healthy controls, B) diabetes mellitus patients, C) chronic urticaria patients and D) Hashimoto's thyroiditis patients.

TABLE I. Obtained values (in ng L^{-1} ± *SD*) for the five serum biogenic amines in healthy controls, diabetes mellitus patients, chronic urticaria patients and patients with Hashimoto's thyroiditis; *SD* is the standard deviation of six replicate determinations; C = healthy controls, D = diabetes mellitus, U = chronic urticaria and H = Hashimoto's thyroiditis patients

Analyte	Controls (n = 20)	Diabetes mellitus (n = 20)	Chronic urticaria (n = 20)	Hashimoto's thyroiditis (n = 8)	*P*-value[a]	*Z*-value[a]
Putrescine	1715±280	230±60	450±50	255±110	<0.0001	C(D,U,H)
Histamine	285±110	505±230	140±60	165±139	<0.0001	D(C,H,U)
Spermidine	855±180	1205±310	1825±580	1165±410	<0.0001	C(D,U,H)
Epinephrine	382±60	490±140	965±510	395±180	<0.0001	U(D,H,C)
Norepinephrine	–	–	700±40	–	–	–

[a]Results of the statistical Kruskal–Wallis test with the multiple-comparison *Z*-value test

of patients with diabetes mellitus, chronic urticaria and Hashimoto's thyroiditis were higher in comparison with those for the healthy controls ($P < 0.0001$; *Z*: C(D,U,H)). The epinephrine levels between chronic urticaria patients and healthy controls, diabetes mellitus and Hashimoto's thyroiditis patients were also statistically significantly different ($P < 0.0001$; *Z*: U(D,H,C)). The chronic urticaria patients had higher concentrations of serum epinephrine compared to those of the controls ($P < 0.0001$). Norepinephrine (a hormone related to stress) was detected only in the serum of chronic urticaria patients.

The findings of enhanced histamine levels in diabetes mellitus patients are in agreement with the work of Gill *et al.*[26] who also found increased histamine concentrations in the plasma of patients suffering from diabetes mellitus (types I and II). Moreover, the studies of Inui *et al.*[27] and of Alkan *et al.*[28] performed on rats and mice, respectively, support the notion that endogenous histamine is involved in the pathogenesis of diabetes.

The detection of norepinephrine only in the serum of chronic urticaria patients is in agreement with literature data that patients with adrenergic urticaria have enhanced levels of serum catecholamines.[29] As there were no pre-analytical patients (or healthy control subjects) prepared for venipuncture, it could be concluded that enhanced levels of serum catecholamines are specific only for patients with chronic urticaria.

A possible reason why the obtained values were in disagreement with those of Mao *et al.*[25] might be the influence of nutritive habits on the level of biogenic amines in body fluids, *i.e.*, blood. Patients with chronic urticaria might have a pseudo-allergic reaction (so-called exogenic allergy) caused by biogenic amines (histamine) from food such as canned fish.[30,31]

In clinical practice, it was recognized that patients with chronic urticaria and Hashimoto's thyroiditis diseases were alike and very often followed each other.[33] This could be one of the reasons why the spectra of these two diseases were similar. In patients with Hashimoto's thyroiditis, autoimmune urticaria might appear and *vice versa*, and for this reason clinicians examined whether Hashimoto's thyroiditis occurs in patients with chronic urticaria.[33,34]

CONCLUSIONS

In this study, the levels of putrescine, histamine, spermidine, epinephrine, and norepinephrine in serum of healthy subjects and patients with different immune-mediated disease were investigated for the first time. It was found that the quantities of serum biogenic amines statistically differed in diabetes mellitus, chronic urticaria and Hashimoto's thyroiditis patients, compared to healthy subjects.

To the best of our knowledge, there is no report on the analysis of the levels of biogenic amines in the serum of patients with chronic urticaria or Hashimoto's thyroiditis. Contrary to expectations, it was found that the histamine levels were not elevated in the sera of chronic urticaria patients, compared to those in normal subjects. Furthermore, it was noticed that the values of norepinephrine and epinephrine were increased in patients with chronic urticaria and hence, it is assumed that the human organism produces higher amounts of these substances as a natural defense against anaphylaxis, and simultaneously suppresses the histamine level. Higher levels of spermidine were noticeable within all three investigated immune-mediated conditions compared to within healthy controls.

The putrescine levels in all three immune-mediated disorders were lower than in healthy subjects. Therefore, observation of the levels of spermidine and putrescine might be used as a non-specific marker that indicates that some health, possibly immune-mediated, disorder is occurring in the body.

The presented results hint on biogenic amines as possible autoimmune disease markers, and some future studies could be designed for closer investigations. The possible influence of nutrition habits on the levels of biogenic amines in blood serum could also be the subject of some future studies.

SUPPLEMENTARY MATERIAL

The results of the calibration and validation of the developed method are available electronically from http://www.shd.org.rs/JSCS/, or from the corresponding author on request.

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ИЗВОД

ОДРЕЂИВАЊЕ БИОГЕНИХ АМИНА LC/DAD МЕТОДОМ У СЕРУМУ ПАЦИЈЕНАТА СА ДИЈАГНОЗОМ *Diabetes mellitus*, ХРОНИЧНА *Urticaria* ИЛИ ХАШИМОТОВ ТИРОИДИТИС

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Биогени амини су саставни део готово сваке ћелије. Хистамин (His), полиамини путресцин (Put) и спермидин (Spd), катехоламини адреналин (Epi) и норадреналин (NE) изоловани су из људског серума методом киселе естракције. Преколонска дериватизација је урађена помоћу данзил-хлорида, а затим је уследила LC/DAD анализа биогених амина у циљу утврђивања разлика у њиховим нивоима код пацијената са дијагнозом *diabetes mellitus*, хронична *urticaria* или Хашимотов тироидитис, у односу на здраве контроле, али и утврђивања њихове функције као могућих маркера за имуно-посредоване болести. За одређивање серумских биогених амина коришћена је метода ретенционих времена. Утврдили смо да постоји статистички значајна разлика у нивоима путресцина и хистамина код пацијената са дијагнозом *diabetes mellitus*, путресцина, хистамина, спермидина и епинефрина код пацијената са дијагнозом хронична *urticaria* и путресцина и спермидина код пацијената са дијагнозом Хашимотов тироидитис, у поређењу са здравим контролама. Норепинефрин је детектован једино код пацијената са дијагнозом хронична *urticaria*. Вредности повратног приноса измерене у контролама

кретале су се у опсегу 85,7–106,7 %. Посматрање нивоа спермидина и путресцина би могло да се користи као неспецифични индикатор који показује да се неки биохемијски, могуће имуно-посредован, здравствени поремећај дешава у телу.

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