Phase Retardation for Dehydrated Human Tears Fluid Samples in Diabetes Mellitus

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Abstract. New analytical capabilities are reported for the V. N. Shabalin – S. N. Shatokhina wedge-shaped dehydration method in the study of biochemical mechanisms of eye diseases development under diabetes mellitus, based on the value of phase retardation of dehydrated samples of human tear fluid. The phase retardation was measured for three sample groups of patients: examined and recognized as healthy patients, patients with undetermined diagnosis of diabetes mellitus, patients with confirmed diagnosis of type 2 diabetes mellitus. The proposed new methodological approach enriches the known morphological methods for the analysis of dehydrated samples of various biological fluids with quantitative parameters over the entire sample area. The approach might prove useful in the research practice of medical and biological fields.

Keywords: wedge-shaped dehydration; tear fluid; phase retardation; phase distribution visualization.

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1 Introduction

An increasing use of biological fluids such as mixed saliva, blood, or tear fluid is observed in recent research to identify significant biomarkers of various diseases. Blood and mixed saliva are required both for the diagnosis of non-communicable cardiovascular or oncological diseases, and for the diagnosis of infectious diseases, such as COVID-19 [1–3]. In the 2000s, researchers discovered that tear fluid also carries information about eye diseases [4]. Tear fluid, being the secret of the lacrimal gland and located directly on the eyeball surface, plays an important role in maintaining its healthy condition and functioning [5]. As for the classification of tears, they are divided into basal, reflex and mental tears. The basal tears role is to constantly keep the cornea moist [6]. Reflex tears occur when foreign particles and substances (onions, perfumes, pepper spray, tear gas) irritate the eyes [7]. Mental tears are caused by emotional stress, joy, anger, physical pain, etc. [8]. Under normal conditions, the volume of tear fluid for a healthy person averages about 5–10 μl, while the level of secretion is about 1.2 μl/min [9].

A great number of methods for the biological fluids and tissues analyses are known [10–13]. These include high performance liquid chromatography (HPLC), ultra high-performance liquid chromatography (UPLC), electrophoresis (SDS-PAGE, CE), enzyme immunoassay (ELISA), mass spectrometry, light scattering and morphology of biological fluids [14]. For the first time in an extensive medical research practice, a comprehensive analysis of the crystalline structures of biological fluids in polarized light was proposed in the 1990s by S. N. Shatokhina in her works on structuring during dehydration and further morphological analysis of the obtained samples of biological fluids [15].

In paper [16] by S.V. Zenin, the tear fluid is viewed and analyzed. The tear fluid represents a complex multicomponent colloidal system that forms specific patterns while being crystallized upon drying. These patterns depend on the tear biochemical composition, surface tension and external environmental parameters. The tear fluid consists of 98 percent water, and the remaining 2 percent includes proteins, lipids, electrolytes, and mucins [17]. In this work, to describe

and analyze the tear fluid, we studied its dehydrated structures. We used wedge-shaped dehydration. Unlike non-wedge-shaped dehydration, this process is controlled and forms specific zones.

The dehydration process proper can be divided into several stages, conventionally distinguished are three of them: a) evaporation of water, b) crystal nucleation, c) crystal structures growth. Figure 1 illustrates the process of the tear fluid dehydration during the formation of different types of its structure [18]. The first stage is water evaporation. The evaporation intensity depends on humidity, temperature, and film thickness. In the course of the crystal nucleation, NaCl ions form primary centers of crystallization, and proteins and mucins act as an anisotropic seed, thereby affecting the shape of crystals. With the crystal structures growth, certain patterns are emerging. Each such pattern is a characteristic type of the structure organization. There are 3 of these types: crystalline, amorphous-crystalline and clumpy-crystalline clumpy-crystalline (Fig. 1) [18]. The 4th column in the Fig. 1 shows images of crystallized tear fluid for each type of organization in polarized light.

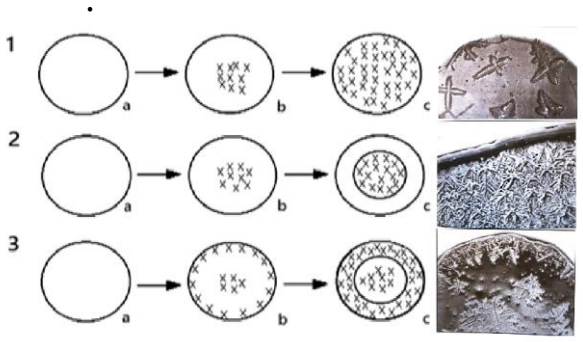


Fig. 1 Types of the tear fluid structure organization: 1 − crystalline, 2 − amorphous-crystalline, 3 − clumpy-crystalline. Explanations of panels (a−c) are given below in the text. The 4th column shows images of crystallized tear fluid for each type of organization in polarized light.

A qualitative analysis of the dehydrated structures of biological fluids proved a high informative value of the detected markers of such common pathological conditions as hypoxia, hyperglycemia, stagnation in tissues, intoxication, ischemia, sclerosis, etc., which form a “mosaic” of typical pathophysiological processes [15]. The dehydrated structures of most biological fluids are polarization-inhomogeneous media. In such structures, the phase retardation value of the light wave and the state of polarization ellipticity change when light passes through them. Analyzing the sample pattern in a polarizing interferometer (in a scheme with crossed polarizers), it is possible to draw conclusions about certain diseases, such as blepharitis, dry eye syndrome, inflammatory and dystrophic eye diseases, including diabetic retinopathy, etc., based on the type of such structures [15, 18]. The phase retardation value is determined by the thickness of the medium and the refractive index for the area under study. The magnitude of the phase retardation can also be different in various parts of the sample and for different diseases.

Morphological studies of biological fluids are mainly a qualitative analysis. By supplementing it with the quantitative measurements results we can expand the analytical resource of the wedge-shaped dehydration method. The purpose of our work was to visualize and measure the phase retardation of dehydrated tear fluid samples for patients without a diagnosis of diabetes mellitus and for patients with the confirmed diagnosis of type 2 diabetes mellitus and to catty out its statistical analysis.

2 Object, Material, and Methods

Dehydrated tear fluid samples achieved by the V. N. Shabalin-S. N. Shatokhina method of wedge-shaped dehydration [15] are glass substrates with tear fluid applied onto them. Reflex tear fluid caused by the mechanical stimulation was noninvasively obtained at the T. I. Eroshevsky Ophthalmoendocrinological Consultative and Diagnostic Division of the Samara Regional Clinical Ophthalmological Hospital. These samples were then dehydrated at a constant room temperature until their complete drying. During the dehydration process, the salt components are drawn together towards the center by surface tension forces, while the protein formations remain at the sample edges. Fig. 2 shows a typical structure of the dehydrated tear fluid sample. In the micrograph, the numbers mark the sample zones. Below is the central (salt) zone, filled with numerous fern-like crystalline structures, and the marginal (protein) zone, which is amorphous. Our measurements were carried out in the shown areas of the obtained samples.

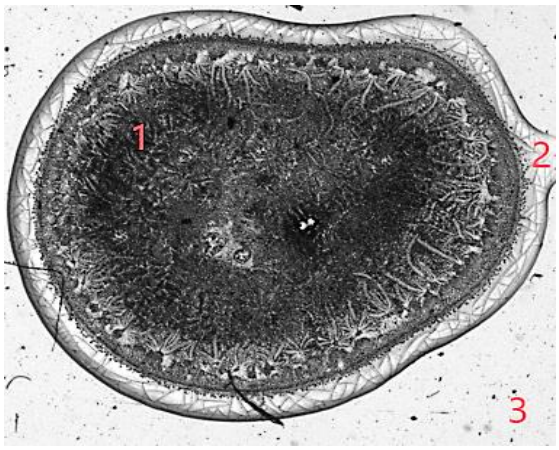


Fig. 2 The typical structure of the dehydrated tear fluid sample in polarized light. The following areas marked with numbers are shown: 1 − central zone (salt structure), 2 − marginal zone (protein substrate), 3 − slide.

Phase retardation visualizer (THORLABS LCC7201B) was used for the measurements. It provides measurement of the phase retardation in the 0 to π range for a wavelength of 633 nm, while the accuracy level of 9.93×10^{-6} rad is ensured. The accuracy of the device is

determined by the interferometric method with the aid of the phase retardation. It consists in the following: a liquid crystal light modulator capable of setting various phase retardations between the arms of the interferometer, is fixed on one of the arms. Thus, the increase in the measurement accuracy during the interference patterns processing is achieved. During the measurement process, about 103 thin dried structures were examined, and phase retardation values ranged from 0.01 nm to 30 nm. Therefore, it can be said that the phase shift visualizer can detect even thin and complex structures.

To measure the phase retardation of the dehydrated tear fluid samples [12], the five-point method was used, often applied in evidence-based medicine. This method consists in the selected parameters measurement at 5 equidistant points (4 points are in the sample marginal (protein) zone and 1point is located in the central (salt) zone). Figure 3 illustrates the application of the five-point method. The described method is recognized for such tasks, as the marginal (protein) area is homogeneous. However, we also tested this method, calculated errors and deviations (at the stage of calculating non-parametric criteria). We also compared the average value of the obtained values using the 5-point method with the value obtained by averaging the phase delay over the entire boundary area (more than 20 points). We obtained relatively similar results for one sample.

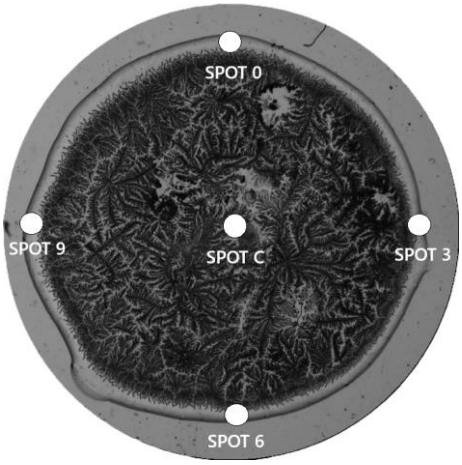


Fig. 3 Estimation of the phase retardation at 5 equidistant points on the surface of a dehydrated tear fluid sample. Polarization microscopy.

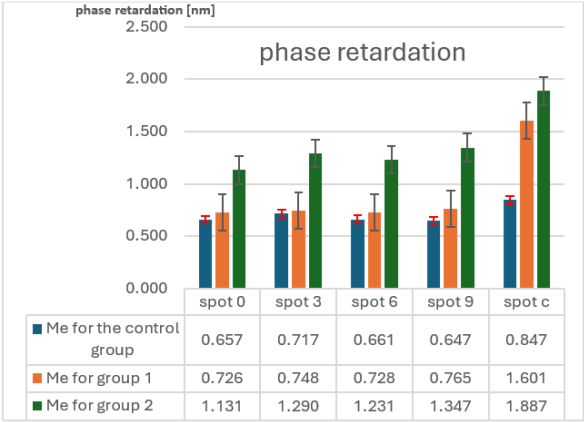
In total, 103 samples were measured (10 samples from the healthy patients group (Reference Group), 68 samples from a group of patients without a diagnosis of diabetes mellitus but with combined dystrophic pathology of the visual organ, i.e. the initial stage of glaucoma, mild myopia and incomplete complicated cataract (Group 1), and 25 samples − from a group of patients diagnosed with type 2 diabetes mellitus with the nonproliferative stage of diabetic retinopathy in combination with dystrophic pathology of the visual organ i.e. glaucoma in its initial stage, mild myopia and incomplete complicated cataract (Group 2), all the measurements were entered in the electronic database.

Statistical data processing in this work was carried out with the subsequent calculation of nonparametric criteria so that to evaluate the reliability of differences between the groups. For each measurement point the median (Me, second quartile), the 25th and 75th percentiles (first and third quartiles), maximum (Max) and minimum (Min) values were found.

There is a lot of nonparametric criteria for comparing two independent samples, whereas a special place belongs to the criteria most frequently applied in biomedical data statistics [4, 19−21], they are the Mann-Whitney *U*-test, the Kolmogorov-Smirnov test and the Wald-Wolfowitz test.

3 Results and Discussion

Basing on the data obtained, a bar graph shown in Fig. 4 was built. It shows the median values for the phase retardation in nanometers (for a wavelength of 633 nm, the phase retardation of π corresponds to 316 nm) for 5 points of three different groups (Reference group, Group 1, Group 2). The groups are marked with different colors, points 0, 3, 6, 9 are located in the sample marginal (protein) area, and the point c is in the central (salt) zone. It can be seen from the presented data, that the phase retardation for Group 2 exceeds this value for the Reference Group more than by 1.5 times. Statistically reliable differences between Group 1 and the Reference Group for this parameter are observed at point C.



	spot 0	spot 3	spot 6	spot 9	spot c
Me for the control group	0.657	0.717	0.661	0.647	0.847
Me for group 1	0.726	0.748	0.728	0.765	1.601
Me for group 2	1.131	1.290	1.231	1.347	1.887

Fig. 4 The value of the phase retardation distribution depending on the measurement point for the three groups of the examined patients.

As a result of the preparatory stage of statistical processing, we did not find a normal distribution for any of the parameters studied. This circumstance determined our subsequent use of only nonparametric tests.

To verify the validity of differences in phase retardation between the groups of patient's nonparametric criteria were used, such as the Mann-Whitney *U*-test, the Kolmogorov-Smirnov test, and the Wald-Wolfowitz test. These criteria were calculated for different pairs of samples: Reference Group − Group 2 and Reference Group joint with Group 1 − Group 2.

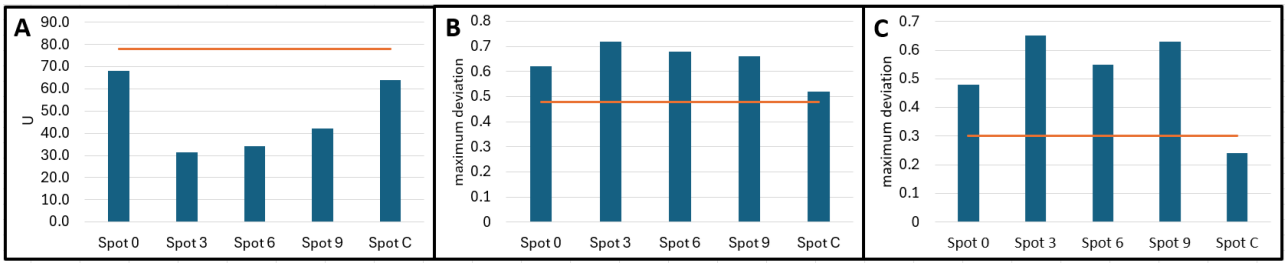


Fig. 5 Graphical representation of the statistical criteria values: (A) the Mann-Whitney *U*-test, (B) the Kolmogorov-Smirnov test for the samples of Reference group and Group 2; (C) the Kolmogorov-Smirnov test for the samples of Reference Group joint with Group 1 and Group 2. The red line on the charts represents the critical value.

For illustration purposes, some nonparametric criteria are presented in the form of bar graphs (Fig. 5). From diagram A, corresponding to the Mann-Whitney *U*-test, it can be seen that the differences are statistically significant, since the value of the phase retardation for each point does not exceed the critical value. In the following two bar graphs, according to the Kolmogorov-Smirnov test, the situation is reversed, the differences are statistically significant if the value obtained is higher than the critical one. In accordance with the Wald-Wolfowitz test, the statistical significance of the differences is also confirmed.

The results of these criteria calculation prove that the differences in phase retardation between the samples of groups of the examined patients are statistically reliable.

4 Conclusion

In the course of the work fulfilled, the distributions of the phase retardation of the tear fluid dehydrated structures were studied for three types of samples: from a group of healthy patients (Reference Group), a group of patients without a diagnosis of diabetes mellitus but with combined dystrophic pathology of the visual organ (Group 1) and a group of patients with a confirmed diagnosis of type 2 diabetes mellitus with diabetic retinopathy in the nonproliferative stage in combination with dystrophic pathology of the visual organ (Group 2).

The phase retardation was measured for 103 studied samples, and an electronic database was formed based on these measurements. It can be seen from the presented data, that the phase retardation for Group 2 exceeds this value for the Reference Group more than by 1.5 times. All the results obtained were statistically processed by 3 different non-parametric criteria (the Mann-Whitney *U*-test, the Kolmogorov-Smirnov test, the Wald-Wolfowitz test), confirming that the phase retardation is an informative value.

Thus, the applied methodological approach based on the phase retardation measurements, can take its rightful place in research practice for analyzing the optical characteristics of biological objects of this type, which is proved by statistically significant differences between groups of examined healthy patients and those with various eye diseases.

It is worth noting that this study does not violate ethical policy. All tear fluid samples were obtained at the Eroshevsky Ophthalmological Clinic, where each patient signs an informed voluntary consent to medical intervention (order of the Ministry of Health of the Russian Federation dated November 12, 2021, number 1051n).

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Disclosures

The authors declare no conflicts of interest in this paper.

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