METHODS

Fluorescent Method for Albumin Assay in Human Aqueous Humour and Tear Fluid

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A new method for albumin fluorophotometry in human ocular fluids (aqueous humour and tear fluid) with a K-35 fluorescent probe is proposed. The method provides measurements of albumin concentration and fluorescence parameters characterising properties of binding centres of the albumin molecule. There was a strong correlation between albumin concentrations determined by fluorescent method proposed by us and by electrophoresis of ocular fluid proteins. This method can be used for the analysis of ocular fluid albumin and the state of the tissue-blood barriers.

Key words: *fluorescent probe*; *albumin*; *antioxidant activity*; *aqueous humour*; *tear fluid*

According to modern concept, disturbances in the permeability of the blood-aqueous barrier and metabolic changes in the aqueous humour play an important role in the pathogenesis of inflammatory and dystrophic eye diseases [7,8,10,14]. Albumin and other aqueous humour proteins actively participate in these processes and the content of albumin in ocular humour changes significantly [5,6,9,12,13]. Function of albumin depends not only on its concentration but also physicochemical properties of this protein [1,4]. Therefore, evaluation of albumin binding capacity in aqueous humour and tear fluid (as the most convenient object) is important for biophysical ophthalmology.

The absence of adequate methods for determination of albumin properties in aqueous humour and tears hampers the progress in this field. The use of fluorescent probes (FP) for these purposes is a perspective approach. FP are small organic molecules, whose fluorescence depends on the conformation state of the protein they are bound to. The advantage of this method is high sensitivity allowing to work with trace amounts of biological material. However, most of FP methods, in particular, in which well known 8-aniline-1-naphthalene-sultanate probe is used, require isolation of albumin from test fluid and independent determination of its concentration. This seems to be unacceptable because of small volumes of aqueous humour (10-50 μl) and tear fluid (10-100 μl) samples and interference with other proteins in recording fluorescence parameters.

A way to solve this problem is the use of highly selective K-35 fluorescent probe (more than 95% of total plasma fluorescence is determined by albumin-bound probe). It was successfully used for the analysis of serum samples from donors and patients with different pathologies. K-35 allows to measure albumin concentration in the plasma [1,2]. The possibility of using K-35 for studying albumin properties in ocular fluids has not been yet investigated.

The aim of the present study was to elaborate express albumin microassay for human aqueous humour and tear fluid using K-35 fluorescent probe.

MATERIALS AND METHODS

Samples of aqueous humour, tear fluid, and blood serum from 53 patients with senile cataract and primary open-angle glaucoma were analysed. The control group comprised 46 healthy subjects (without ophthalmo-

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pathology) aged 45-75 years, tear fluid and blood serum samples were collected.

Aqueous humour (30-80 μl) from the anterior eye chamber was obtained during surgical intervention by paracentesis with an insulin syringe. Antiglaucoma drugs were ceased 1 day before the surgery. Tear secretion was stimulated by inhalation of ammonia vapour and tear fluid (20-50 μl) was collected from the bottom of the conjunctival sac with a capillary without preliminary anaesthesia. Blood was drawn from the cubital vein and the serum was prepared at room temperature using a routing procedure. The samples were put into plastic vials and stored at -18°C.

Total protein content in aqueous humour was determined by the Lowry method in 10-μl samples [11]. Protein fractions in biological fluids were analysed by cellulose acetate electrophoresis [3] with modifications allowing to detect protein fractions in fluids with low protein content. To this end, aqueous humour was concentrated and 3 drops of the concentrate (total volume 10 μl) were applied to the start point of electrophoretic paper. Densitometry was performed in transmitted light on an Acta Service densitometer at 620 nm.

A Zond-Al'bumin assay kit (Zond Medical Center, Moscow) was used for the analysis of albumin properties. This kit includes three reagents: 1 — buffer solution (pH 7.4); 2 — K-35 probe; and reagent 3 for determination of albumin content in the serum.

Effective (EAC) and total albumin concentrations (TAC) in the serum were determined with this kit according to the manufacturer protocol.

Before testing aqueous humour and tear samples, reagent 2 was diluted (1:10) with reagent 1. Tear fluid was also diluted with this solution if necessary. Calibration curve for albumin was constructed using standardized serum samples (GSO 6120-91 of types O0 and N3).

The data were processed statistically using Exel 7.0 software. Differences between groups were evaluated by Student's t test and nonparametric Wilcoxon test at α=0.05.

RESULTS

Fluorescent albumin assay for the serum and plasma served as the basis for our measurements [1]. TAC and EAC can be evaluated by this method. TAC, i.e. mass albumin concentration in test sample corresponds to its concentration in the plasma measured by routine biochemical methods [2]. $TAC=A\times F_3$, where F_3 is the intensity of K-35 fluorescence in the sample in the presence of reagent 3, and A is a coefficient which does not depend on albumin physicochemical properties (K-35 was used in a saturating concentration with respect to albumin binding sites). $EAC=B\times F_2$, where F_2 is the intensity of K-35 fluorescence in the sample at pH 7.4 (at the same concentration of reagent 2), and B is a coefficient which depends on both the albumin concentration and parameters of albumin binding sites under physiological conditions. The EAC/TAC ratio (B/A) does not depend on albumin concentration and is determined by physicochemical properties of albumin.

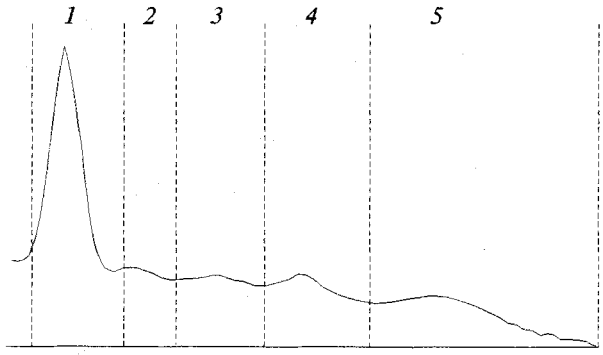
Measurement of TAC and EAC in aqueous humour and tear fluid includes the following stages. Reagent 2 (50 μl) was diluted 1:10 with reagent 1 and added to aqueous humour or tear sample. Fluorescence intensity was measured at emission and excitation wavelengths of 520 and 420 nm, respectively. EAC was calculated by the intensity of fluorescence using the calibration curve. After addition of reagent 3 (25 μl) to the sample fluorescence was measured again under the same conditions and TAC was calculated by the calibration curve. For construction of the calibration curve the samples with known EAC and TAC was adjusted to concentrations of 10-1000 μg/ml.

To validate albumin concentrations in aqueous humour determined with fluorophotometry they were com-

TABLE 1. Parameters of K-35 Fluorescence in Tear Fluid, Aqueous Humour, and Serum from Patients with Eye Diseases and Normal Subjects ($M\pm m$)

Groups	EAC	TAC	EAC/TAC
	g/liter		
Patients			
Serum (n=53)	20±0.74*	40.8±1.05	0.49±0.03*
Aqueous humour (n=53)	0.05±0.015	0.14±0.02	0.34±0.025
Tear fluid (n=30)	0.35±0.05*	1.26±0.22	0.28±0.025*
Controls			
Serum (n=18)	33±1.83	44±1.15	0.75±0.05
Tear fluid (n=46)	0.83±0.08	1.48±0.25	0.42±0.03

Note: *p<0.05 compared with the control group.



Fraction	Proportion, %	Protein content, mg/ml
1. Albumin	51.0	0.107
2. α1	4.64	0.009
3. α	27.84	0.016
4. β	14.4	0.030
5. γ	22.1	0.046

Total protein=0.21 mg/ml
Albumin/globulin=1/04

Fig. 1. Densitogram of aqueous humour from patient K. (age 62) with developed open angle glaucoma and high intraocular pressure in the right eye. Numbers above peaks correspond to protein fractions.

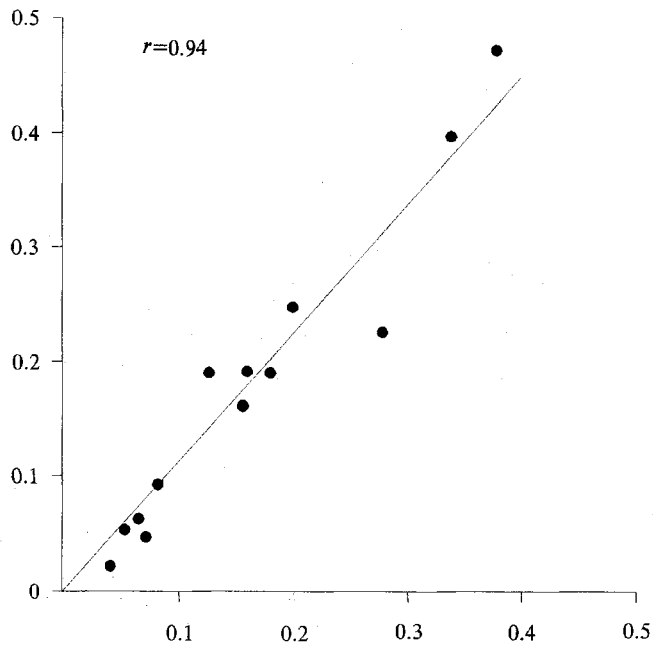


Fig. 2. Correlation between albumin concentrations (g/liter) in aqueous humour determined by electrophoresis (abscissa) and fluorescence method (ordinate).

pared with electrophoresis data. Figure 1 shows a representative densitogram. Five protein fractions were identified on the densitogram obtained with a modified electrophoretic technique for concentrated aqueous humour (Fig. 1). Albumin concentration was calculated from densitogram and total protein measurements.

A strong correlation was proved between the albumin concentrations (TAC) determined by electrophoresis and fluorescent method (Fig. 2). Regression was plotted through coordinate origin because the ordinate of the intersection point practically did not differ from zero. Standard deviation from the regression curve was 0.0012 g/liter, the slope was 1.12. This discrepancy can be due to different compositions of the serum and aqueous humour (in particular, reduced proportion of nonalbumin components in aqueous humour).

Thus, the two applied methods for evaluation of albumin concentration in biological fluids yield the same results.

EAC and EAC/TAC ratio are sensitive to conformational changes in albumin molecules during ophthalmopathy (Table 1).

In patients with glaucoma and cataract EAC and EAC/TAC were significantly reduced compared with normal subjects ($p<0.05$). In both healthy subjects and patients with eye diseases ETC/TAC in the tear fluid was lower than in the serum, and in the aqueous humour this parameter was intermediate.

Thus, properties of binding sites in albumin molecule assessed by fluorescent method vary depending on the state of the organism and blood-aqueous barrier. The properties of ocular fluid protein can be evaluated with the fluorescent method and used for assessment of the state of tissue barriers in the eye.

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