Human Eye Lens Fluid Analysis Using High-Performance Liquid Chromatography-LED-Induced Fluorescence to Explore Cataract: A Pilot Study

Sulatha V. Bhandary[1], Sphurti S. Adigal[2], Jijo Lukose[2], Reena V. John[2], Alisha Rizvi[1], and Santhosh Chidangil[2*]

[1] Department of Ophthalmology, Kasturba Medical College (KMC), Manipal Academy of Higher Education, Manipal 576104, Karnataka, India

[2] Centre of Excellence for Biophotonics, Department of Atomic and Molecular Physics, Manipal Academy of Higher Education, Manipal 576104, Karnataka, India

***e-mail:** santhosh.cls@manipal.edu

Abstract. Cataract is the common cause of reversible blindness and visual impairment. It is common in low socioeconomic groups in developing countries. This paper describes the application of an assembled High-Performance Liquid Chromatography-LED-Induced Fluorescence (HPLC-LED-IF) system to study the protein profiles of lens fluid samples from cataract patients with and without diabetes. The HPLC-LED-IF system assembled in our laboratory was used to record protein profiles of lens fluid samples obtained from volunteers having cataract with diabetes and without diabetes who have undergone cataract surgery. Statistical analyses such as Descriptive Statistics and Principal Component Analysis (PCA) were conducted to observe any kind of changes in the protein pattern of the two classes of samples. High-Performance Liquid Chromatography combined with LED-Induced Fluorescence (HPLC-LIF) generated lens fluid protein profile data of the two categories (cataract with and without diabetes) of samples have shown considerable changes in their pattern. These changes are confirmed by Descriptive Statistics and PCA. Protein profiles of lens fluid using an assembled HPLC-LED-IF system have shown significant differences in relative intensities of crystalline proteins of cataract with diabetes and without diabetes. UV absorption and fluorescence spectroscopy of the lens fluid have shown valuable information on the absorption and fluorescence intensity variations. The origin of the changes has to be further studied to get more information on this observation.

Keywords: cataract lens fluid; UV-absorption; fluorescence; HPLC-LED-IF; protein profile.

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1 Introduction

Cataract is the cause of visual disability worldwide, affecting more than half of all blindness conditions in the world [1, 2]. It is a disease of the eye, which is manifested as an opacification of the crystalline lens that ultimately results in minimizing visual perception [3, 4]. Each year, the number of patients with cataract increases exponentially. The mechanism of cataract may be due to conformational changes, aggregates formation, or

changes in the backbone of crystalline proteins. Moreover, the changes in tyrosine and tryptophan residues play a pivotal role in cataract formation [5]. The prevalence of this disease is observed to be more in diabetic patients than in normal adults [6]. The report says that cataract occurs more frequently at an earlier age in diabetic individuals [2]. According to the latest world health organization (WHO) reports, more than 150 million people have diabetes mellitus with predictions showing a further enhancement by 2025 [7]. Currently for cataract, the only treatment available is lens replacement surgery.

In the United States, the total cost of treating diabetes-related diseases is reported to be 10% of all healthcare expenditures [8]. This has high critical significance in developing countries like India since this country has more people with diabetes than any other nation in the world, per the estimates from the International Diabetes Federation (IDF). The reports suggest that there are currently above 66 million people suffering from diabetes in India [9]. High blood sugar levels can lead to the swelling of the eye lens, which may damage the lens leading to protein buildup on the lens. This can also be a reason for cataract formation in Indians at an earlier age. Indian population tends to develop cataract at 14 years younger than their counterparts in the US. The reports suggest that, about 74% of adults over 60 years are struggling with cataract or have performed cataract surgery [10].

Currently, light scattering methods are used for diagnosing cataract and these methods help in detecting the defects in the lens structure [11]. However, there are no well-documented scientific reports on the cataractogenic mechanisms of cataract development, which are still being investigated. To explore the utilization of lens fluid and to understand more about the development of cataract as well as its correlation with diabetes condition, it is crucial to know the changes in physicochemical events that lead to cataract formation. In view of this, the disposed materials (lens fluid) after the phacoemulsification procedure can be studied with spectroscopic techniques and separation methods like high-performance liquid chromatography for the characterization of the samples of the two categories of cataract cases having cataract with diabetics and without diabetic conditions.

Meanwhile, proteomic analysis is being largely used to identify the changes in protein levels in cataract patients. Proteins are essential mediators in all physiological processes, and they give valuable information on biomarkers for various diseases. Proteomic methods identified Multiple proteins in human aqueous humor (AH) of the cataract lens disease [12−15]. Recently, a study has used label-free nanoflow ultra-high-performance liquid chromatography-tandem mass spectrometry (n-UPLC-MS/MS) quantitation to analyze the proteins in single-risk and double-risk of the AH samples [6]. To investigate the mechanism of cataract formation, highly sensitive proteomic methods can probably be useful by using a small quantity of samples, thereby favoring the methodological approach for these investigations [6].

Our earlier studies have discussed the protein profile analysis using HPLC-LIF of different clinical samples (body fluids) like, serum, saliva, vaginal wash, etc. [16−18]. These studies have shown that this method can be highly useful for disease diagnosis. Recently, studies have been extended for analyzing the body fluids for cardiovascular and ophthalmological disease diagnosis by replacing the existing excitation source, 257 nm laser, with a low-cost light emitting diode (LED) with 278 nm emission for detecting the protein eluted from the separation column of the instrument. In these studies, we have discussed the HPLC-LED-IF system's performance in the diagnosis of myocardial infarction, dry eye, and primary open-angle glaucoma [19−21]. In these studies, we have evaluated the system's performance by studying the limit of detection of different protein markers for the diagnosis of diseases such as myocardial infarction, dry eye, and primary open angle glaucoma. In the present scenario, in clinical diagnostic practices usually one or two protein markers are monitored. This approach may not be useful for early detection, prognosis, therapy follow-ups, etc. However, in protein pattern analysis, one can observe how the pattern generated by multiple proteins is useful for clinical application.

To the best of our knowledge, protein profile analysis and comparison of diabetic and non-diabetic cataract by collecting the lens fluid have not been explored. This hyphenated method (HPLC-LED-IF) can probably help to explore the mechanism of cataract formation using the highly sensitive and cost-effective method mentioned here. In this study, efforts have been made to investigate the extracted cataract lens fluid from diabetes and non-diabetes patients using HPLC-LED-IF-based protein profiling technique.

2 Material and Methods

2.1 Sample Collection

Institutional Ethics Committee (634/2021) approval has been obtained for this study. Informed consent was taken from the volunteers who participated in this investigation. This study was performed on eye lens fluid samples obtained from 40 patients who have undergone cataract surgery. The sample details are given in Table 1. Two groups, 22 non-diabetics with cataracts and 18 diabetes with cataracts were considered for the study. Samples were collected after phacoemulsification surgery from Operation Theatre of the Kasturba Hospital, Manipal, Karnataka, India.

2.2 Sample Processing

The collected samples were further homogenized using a mechanical homogenizer to crush the lens pieces that remained in the fluid kept in ice cooling condition. Then the samples were centrifuged at 1396 g (5000 rpm) for 5 min and the supernatants were slowly pipetted out.

Table 1 Sample details gender and age-matched (above 45).

Gender	Cataract with non-diabetes (12 Male, 10 Female)			Cataract with diabetes (10 Male, 8 Female)		
	Alcohol consumption	Smoking	None	Alcohol consumption	Smoking	None
Male	2 (15, 16)	3 (18−20)	7	3 (25−27)	4 (31−34)	3
Female	None	None	10	None	None	8

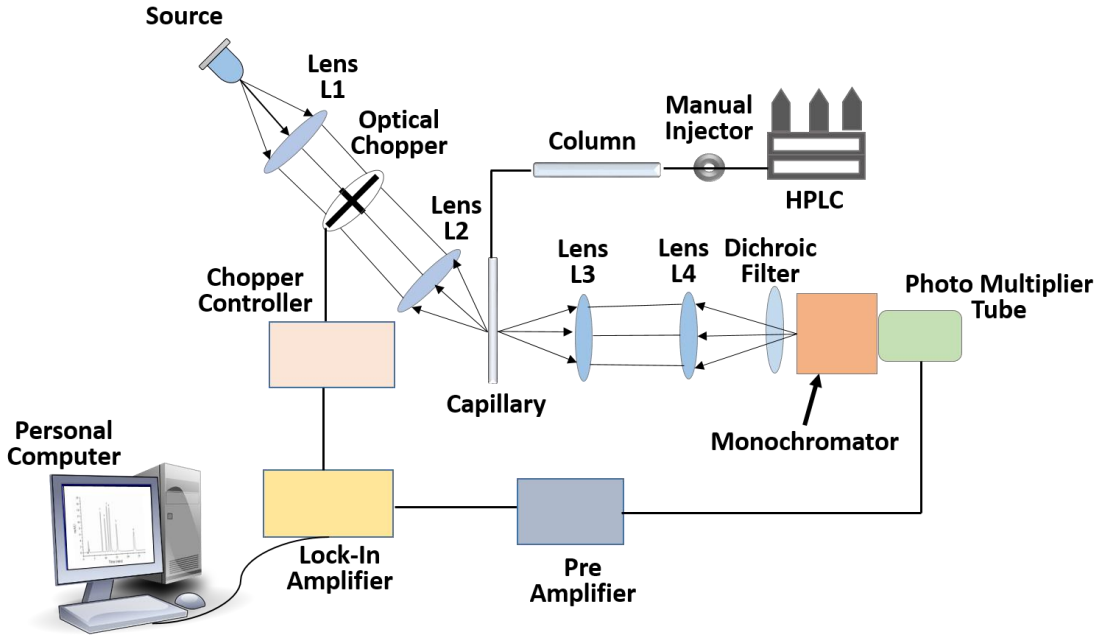

Fig. 1 Schematic of HPLC-LED-IF system.

After centrifugation, each lens fluid sample (90 µL) was diluted in 10 µL HPLC grade water and stored in −80 °C deep freeze until analysis.

2.3 UV-VIS Absorption and Fluorescence Study

UV-VIS absorption measurements were studied using JASCO UV/Visible Spectrophotometer V-650. The experiment was performed to select the appropriate excitation wavelength for recording protein profiles of lens fluid as well as to get information about the absorption and fluorescence characteristics. Fluorescence spectra were recorded using JASCO Spectrofluorometers FP 8500.

2.4 HPLC-LED-IF Setup

The HPLC-LED-IF setup is composed of two parts, the HPLC unit for the protein separation and a locally built LED-Induced fluorescence (LED-IF, 278 nm) system for the detection of the separated proteins (Fig. 1). In the study 300SB-C8 column (4.6×250 mm, 5 mm) of pore size 300 Å was used to separate the proteins in lens fluid from cataract samples. For a more comprehensive understanding of the experimental setup's construction and operation, additional information can be found in the referenced source [19].

The chromatograms were recorded using mobile phase A - HPLC grade water with 0.1% Tri Fluoro Acetic acid (TFA) (A), and mobile phase B - Acetonitrile with 0.1% TFA (B). The gradient runs for separating tear fluid proteins are (i) A: 90% to 65% and B: 10% to 35% in 15 min, (ii) A: 65% to 60% and B: 35% to 40% in 15 min, (iii) A:60% to 45% and B: 40% to 55% in 25 min, and (iv) A: 45% to 25% and B: 55% to 75%. The above gradient was selected by performing several runs by varying the concentration of mobile phase A and mobile phase B, till obtaining a maximum number of peaks in the chromatogram. The column was cleaned with gradient eluent A for 10 min after each run. The optimized flow rate for all runs was 0.2 ml/min.

2.5 Eye Lens Fluid Analysis

The lens fluid chromatograms were preprocessed by correcting the baseline and smoothing technique to reduce the fluctuations of frequency in LED power and PMT dark current using GRAMS/32 (Galactic Inc., USA) software. The protein profiles were calibrated to reduce the peak position shifts, by assigning the mean values for the common protein peaks in all the chromatograms along the time scale [22]. A descriptive

statistics study was performed to identify the differences between cataracts with and without diabetes. In this study, box plots were drawn based on mean and standard deviation values to determine the variations in the different regions of both cataracts with diabetes and cataracts without diabetes. PCA was performed to show the clear-cut discrimination between cataracts with and without diabetes. In PCA, the mean of all the protein profiles is calculated and then subtracted from each protein profile data. This difference in protein profiles gives variations in each sample from the mean, that are subjected to PCA [20]. The two main factors, PC1 and PC2, in a plot differentiate between cataracts with and without diabetes. Both analyses (descriptive statistics and PCA) were performed using Unscrambler X (version 10.4 CAMO, Norway) software [23]. In this method, factor 1 and factor 2 were used to discriminate between cataracts with diabetes and without diabetes.

3 Results

The spectra shown in Fig. 2(a) are the average absorption and fluorescence spectra of lens fluid from 10 cataract patients with diabetes and 10 without diabetes respectively. From Fig. 2(a), it is evident that the crystalline lens fluid's absorption is at 280 nm.

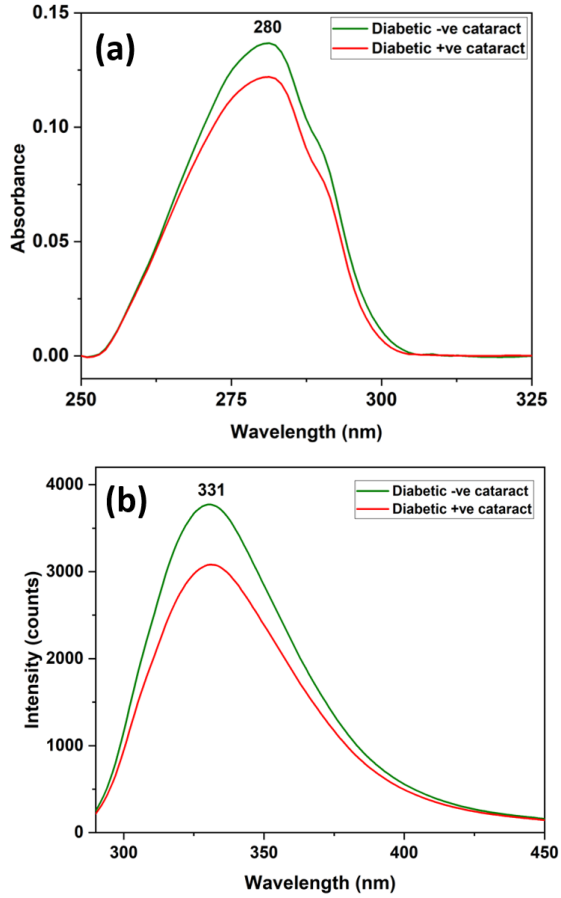

Fig. 2 (a) UV-Vis absorption spectra of eye lens fluid with diabetic -ve cataract and diabetic +ve cataract and (b) Fluorescence spectra of diabetic -ve cataract and diabetic +ve cataract eye lens fluid at 280 nm excitation.

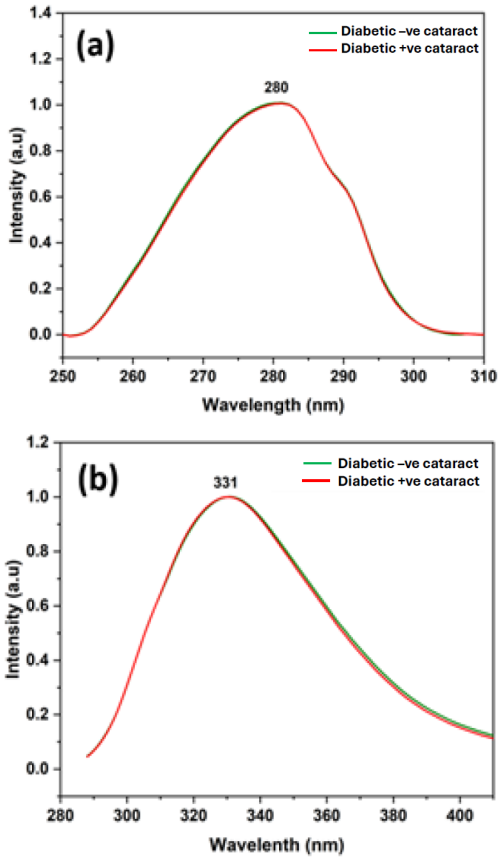

Fig. 3 (a) Normalized UV-Vis absorption spectra of diabetic -ve cataract and diabetic +ve cataract and (b) Normalized fluorescence spectra of cataract eye lens fluid with diabetic -ve cataract and diabetic +ve cataract with 280 nm excitation.

In view of the absorption at the 280 nm region, emission spectra of lens fluid have been recorded at 280 nm excitation to investigate the fluorescence properties of the samples. A fluorescence emission at ~ 331 nm has been obtained for both samples under the excitation wavelength of 280 nm (Fig. 2(b)). The study showed that the absorption and fluorescence intensities were reduced considerably in cataract lens fluid samples with diabetes. The changes may be attributed to the depletion of tryptophan [11]. Peak normalization was also applied to the both the spectrum to facilitate the comparison between the two groups. Upon visual inspection and analysis of the normalized spectra, no significant shifts or discernible differences were observed between diabetic positive and negative cataract groups.

The average of all preprocessed eye lens fluid chromatograms with the entire protein elution time region is shown in Fig. 4(a). Box plots from Fig. 4(b) show considerable variations among cataracts with and without diabetes for the protein elution time region 1876 s to 3690 s. The lens fluid chromatograms were further subjected to PCA analysis for the protein elution time region from 1876 s to 3725 s. Fig. 5 gives PC1 vs PC2 cluster plot that shows a clear differentiation between diabetic -ve cataracts and diabetic +ve cataracts.

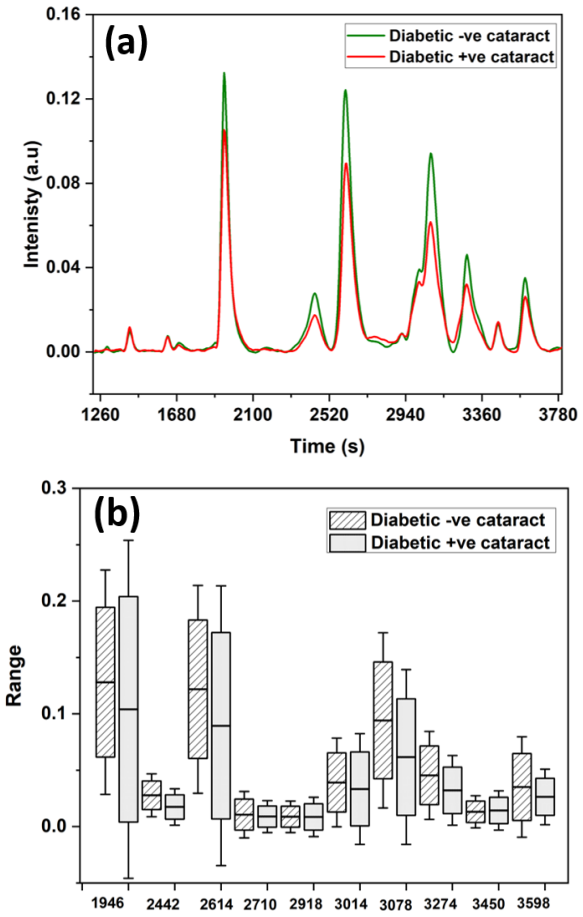

Fig. 4 (a) Average lens fluid chromatogram of diabetic -ve cataract and diabetic +ve cataract (b) Descriptive statistics results of diabetic -ve cataract and diabetic +ve cataract in the protein elution time region 1876 s to 3725 s.

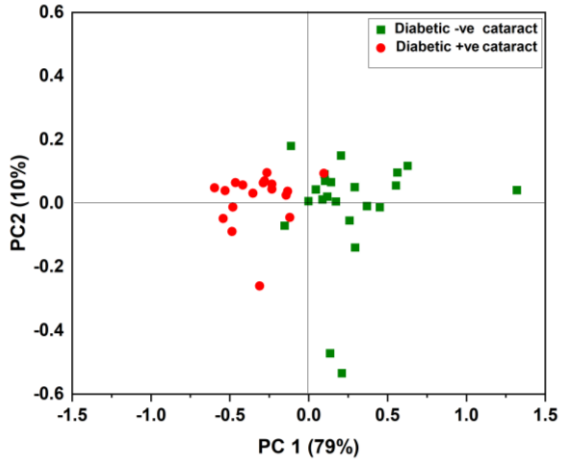

Fig. 5 PC1 vs PC2 cluster plot of diabetic -ve cataract and diabetic +ve cataract.

4 Discussion

The eye lens has the highest percentage of protein content (~38% of total mass) as compared to any other tissue in the human body. Water-soluble crystalline proteins form a major part of the eye lens (more than 90% of total dry mass). Eye lens contains three kinds of crystalline proteins termed α, β, and γ crystallins. Irrespective of the high stability of crystalline proteins, molecular changes accumulate in the proteins over time due to various exogenous factors such as aging, environmental factors, diseases such as diabetes, etc. These changes can ultimately lead to protein denaturation, aggregation, and misfolding, which may result in cataracts [6].

Absorption and fluorescence spectra have shown significant intensity variations between cataracts with and without diabetes in our study. Though the study has shown the differences in the absorption and fluorescence intensity, it does not provide detailed information on various components present in cataract lens fluid that can show fundamental changes between two classes of samples. Therefore, a sensitive and reliable system like HPLC-LED-IF has the highest potential for diagnosing different eye diseases like our earlier studies [19−21].

The protein profiles of lens fluid with cataract with -ve diabetes and cataract with +ve diabetes have shown significant peak intensity variations. Descriptive statistics also showed considerable differences among these two groups in the protein elution time region from 1876 s to 3690 s. Further statistical analysis (PCA) proved that the HPLC-LED-IF system is capable of differentiating between cataracts with and without diabetes. It showed discrimination among these two groups with a total percentage of variance of 89%. To provide the exact identity regarding the protein profile signatures displayed in HPLC analysis, a co-injection technique in HPLC or mass spectrometry of the eluted portions may be required. The number of different disease markers and their relative intensities may vary under different stages of the disease, so identifying each one of them, and their concentrations are very complex and time-consuming. It is not at all necessary when we use the present approach discussed in the manuscript for the discrimination of cataracts with the above two conditions. Thus, the present work is focused on protein pattern changes under cataracts with and without diabetes.

5 Conclusion

HPLC-LED-IF technique was used for investigating the protein profile patterns in the lens fluid collected from diabetic and non-diabetic individuals. Protein profiles of diabetic and non-diabetic cataract lens fluid samples have shown considerable variations. Descriptive statistics results of lens fluid of cataracts with diabetes and without diabetes showed considerable differences. Further PCA analysis differentiated cataracts with diabetes from cataracts without diabetes by analyzing the protein patterns generated by the HPLC-LED-IF system with an 89% total percentage of variance. In our study, we could collect only limited samples for the experiments. Lens fluid samples can provide reliable information about diabetic conditions which is not yet explored. Therefore, to confirm the changes between diabetic cataract and

non-diabetic cataract patients, and to explore the exact mechanism, more experiments are required by collecting a greater number of lens fluid samples.

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Declaration of Interest Statement

The authors are not involved with any organization or entity with a financial interest or financial conflict of interest associated with the manuscript.

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