High performance liquid chromatography analysis of tear protein patterns in diabetic and non-diabetic dry-eye patients

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PURPOSE. *To analyze and compare the high performance liquid chromatography (HPLC) runs of tear proteins from diabetic (DIDRY) and non-diabetic (DRY) dry-eye patients, and healthy subjects (CTRL). The patterns were analyzed using multivariate statistical methods.*

METHODS. *Tears (total 56 eyes: CTRL: n=16, DIDRY: n=21, and DRY: n=19) were analyzed by HPLC, using a size-exclusion column with an eluent of 0.5 M sodium phosphate buffer. The patients were primarily grouped according to the results of the basic secretory test (BST) in combination with subjective symptoms such as burning, foreign body sensations, tearing, and "dryness" of the eyes. Patients with BST values ≤ 10 mm/5 min plus two subjective symptoms were grouped as dry-eye patients. Before statistical analysis, each HPLC run was quantitatively analyzed using ScanPacK software (ScanPacK, Göttingen, Germany), and a data set was created from each HPLC run. The data were then analyzed by multivariate analysis of discriminance.*

RESULTS. *The HPLC patterns of CTRL, DIDRY and DRY were significantly different (Wilks' lambda: 0.0209; p<0.01). The area of the sIgA peak was significantly smaller (p<0.05) in dry-eye tears than controls. There was a good correlation between the extent of separation in the multivariate analysis and the BST value (r = -0.71). Classification of all samples resulted in 98% correct assignments.*

CONCLUSIONS. *The analysis of HPLC patterns and subsequent statistical evaluation are useful for the detection of dry eyes. The HPLC method and the statistical routines described allow a shorter analysis time than electrophoresis. HPLC analysis in combination with statistical analysis can be used as a diagnostic tool for the detection of dry eyes, and also improves the quality of analysis of disease-associated tear proteins in clinical research. (Eur J Ophthalmol 2001; 11: 19-24)*

KEY WORDS. *Dry-eye, Tear proteins, Tears, HPLC, Diabetes mellitus*

INTRODUCTION

Biochemical investigation of tear proteins is important for understanding tear deficiencies, contact lens incompatibilities, tear film instabilities and several other eye diseases. Tear production in the lacrimal glands is influenced by neurotransmitters, viruses, and hormones (1-6). "Dry-eye" describes a disease with various symptoms resulting from aqueous, mucin or lipid deficiency. Tears of healthy persons contain up to 60 different proteins (7). Some of these are also present in serum while others are tear-specific. The molecular weights of these proteins range from approx. 12 kDa (β-2-microglobulin) to 900 kDa (IgM) (8, 9).

New clinical tests for the diagnosis of dry-eye such as ELISA and electrophoresis of tear proteins give very promising results and quantitative analysis of tear proteins could become the most important diagnostic tool in dry-eye patients (1). Despite this, the clinical diagnosis of dry-eye is usually based on mea-

surement of tear-film break-up time (BUT), Schirmer's test and the basic secretory test with local anesthesia (BST). These are the most widely used to establish a diagnosis. However, they correlate poorly with each other and with the course of the disease (10, 11).

In a recent paper we reported that one-dimensional electrophoretic separation of tear proteins with subsequent digital analysis and multivariate statistical analysis can serve as a diagnostic tool for detecting dry-eyes (12). We also showed that the tear protein patterns in diabetic patients suffering from dry-eye syndrome differ significantly from those of healthy subjects or non-diabetic dry-eye patients (12). Gel permeation chromatography by high performance liquid chromatography (HPLC) for analysis of tear proteins has been reported (13-16). This technique can quantitatively analyze the tear proteins in small volumes in a fast and reliable manner. The present study used gel permeation chromatography by HPLC followed by a complex multivariate statistical procedure. This technique has been successfully employed in *myasthenia gravis*, Graves' disease, experimental uveitis, and the analysis of electrophoretic separations of tear proteins (12, 17-20). The procedure allows the analysis even of complex HPLC runs.

The present study was designed to analyze the utility of HPLC runs of human tears in combination with subsequent analysis techniques for the detection of dry eyes.

PATIENTS AND METHODS

Patients and tears

Tears were obtained from 56 eyes: 19 non-diabetic patients with dry-eye symptoms (DRY), 21 diabetic patients with dry-eye symptoms (DIDRY), and 16 healthy subjects (CTRL). The tears were sampled using a 5-µl glass capillary, taking great care not to touch lid margins and eye lashes. To exclude individual differences, tear collection was done by one examiner only. If the lid margin and/or eye lashes were touched the patient was not included in the study. The tears (sample volume abt 5 µl) were immediately stored at –20°C until use.

The tear-film break-up time (BUT) and the basic secretory test (BST) were performed. Each patient's his-tory was taken. The initial clinical diagnosis of dry-eye was based on the BST value and the presence of subjective symptoms such as burning, foreign body sensations, tearing and "dryness" of the eyes: patients were classified as "dry-eye" with a BST value ≤ 10 mm/5 min plus two subjective symptoms.

Biochemical procedures

Tear samples were centrifuged at 12000 g for 3 to 5 min before analysis. The HPLC system used was the Model 2800 (BIORAD, Munich, Germany) with the BioDimension UV/VIS monitor attached (Biorad, Munich, Germany). A size exclusion column (Bio-Silect SEC 250-5; BIORAD, Munich, Germany) was used with an eluent of 0.5 M sodium phosphate buffer (pH 6.0). Two µl of tears were diluted in HPLC buffer (sodium phosphate), injected in a 10 µl sample loop, and measured at 280 nm for 20 min.

The HPLC system was controlled by the Value Chrome chromatography software (Biorad, Munich, Germany) and the data was obtained from the monitor by the BioDimension Control and Reduction Software (Biorad, Munich, Germany). For further analysis the data was exported from the BioDimension software to the quantification software ScanPacK (Biometra, Göttingen, Germany). A data set was created from each HPLC run. Known standards were injected (SEC column protein standards; Biorad, Munich, Germany) for calculation of the molecular weights of peaks.

Calculation

The data were analysed by ScanPacK (Biometra, Göttingen, Germany) which can also be used for densitometric data of electrophoretic separations and has been described elsewhere in detail (21-23). For each HPLC run a data file was created showing the intensity (O.D. values as 8-bit numbers) against the retention time. All peak parameters (height, area under the curve, molecular weight, retention time etc) were calculated. A data vector with 70 variables was created for each HPLC run. Each variable corresponds to 1/70 of the retention time and the value of the variable is built by the mean intensity of all data in this region of the retention time. These data vectors were compiled into a database for subsequent calculations.

Multivariate analysis of discriminance

Each data file was assigned to only one clinically predefined group: CTRL, DIDRY, and DRY. Based on the data vectors created by ScanPacK, a multivariate analysis of discriminance was done. Analysis of discriminance can test whether the data vectors of the groups belong to the same population or whether they are significantly different from each other. In contrast to other standard procedures such as t-tests where each variable would be tested against another single variable, multivariate analysis of discriminance compares the whole set of variables at the same time allowing even complex comparisons.

Discriminant function analysis can also be used to classify the data for diagnostic purposes. It calculates the statistical probability of an unknown HPLC run belonging to which group. The calculation procedures have been described elsewhere in detail (17, 24-26). All statistical calculations were done by Statistica (StatSoft, Tuscon, Arizona, USA).

RESULTS

Tears were analyzed by gel permeation chromatography with HPLC. Figure 1 shows a typical HPLC run of the DRY group at 280 nm. The main peaks in the HPLC runs were identified by comparison with known standards and by checking HPLC elution fractions by sodium-dodecylsulfate gel electrophoresis (not shown). The peak identification was in accordance with the literature (14-16).

The following main peaks were detectd: sIgA (peak 1; tear specific IgA), albumin and lactoferrin (peak 2; LACT), tear lipocalin (27, 28) or tear-specific pre-albumin (peak 3 and 4, TSPA), and lysozyme (peak 5 and 6, LYS). Albumin and lactoferrin were eluted in one peak. Additionally, an IgG peak was found in some tears. The separation by HPLC was very stable. The retention time of any one peak showed a SD of only 10 s during the whole period of the experiments.

Figure 2 shows the areas of the main peaks in all clinical groups. Only the sIgA peaks (No. 1) were significantly different in healthy subjects and the dry-eye syndrome patients (DRY and DIDRY groups, p < 0.05). The area of the sIgA peak was significantly smaller (p<0.05) in dry-eye tears than controls. There was no
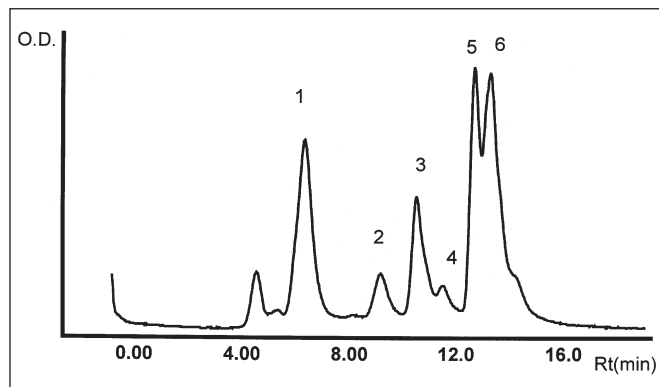

Fig. 1 - *HPLC run of a tear sample of the non-diabetic dry-eye (DRY)group. O.D. (optical density) was plotted against the retention time (Rt). The sample time was 20 min. 1) sIgA. 2) Lactoferrin and albumin. 3 and 4) Tear lipocalin or tear specific pre-albumin (TSPA). 5 and 6) Lysozyme.*
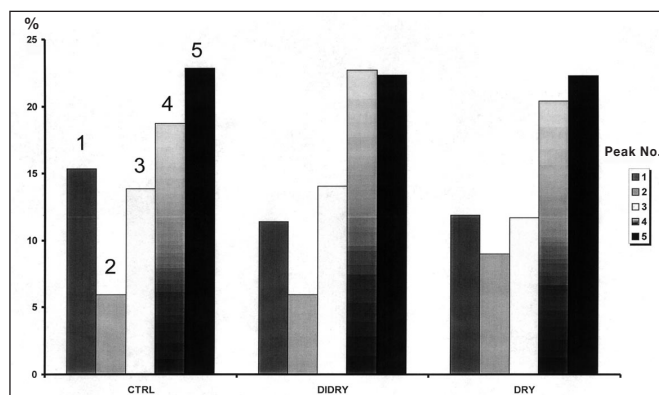

Fig. 2 - *Analysis of the main HPCL peaks of human tear proteins. The average areas under the curve for each group (CTRL, DIDRY, and DRY) were plotted against the peak number. 1) sIgA. 2) Albumin, lactoferrin. 3 and 4) Tear lipocalin or tear-specific pre-albumin (TSPA). 5) Lysozyme.*

significant difference in the average number of peaks per HPLC run between all groups.

Subsequent analysis included the raw data of the HPLC run. Thus, identification of a single peak in the HPLC run is meaningless for this kind of analysis. As described above, for each HPLC run a data vector with 70 variables was built containing the mean O.D. values for each retention time and multivariate analysis of discriminance was done on this. The HPLC runs of all groups were significantly different (Wilks' Lambda: 0.0209; p<0.01).

Figure 3 shows a plot of the canonical roots of the analysis of discriminance in the discrimant space. This means that the greater the distance between
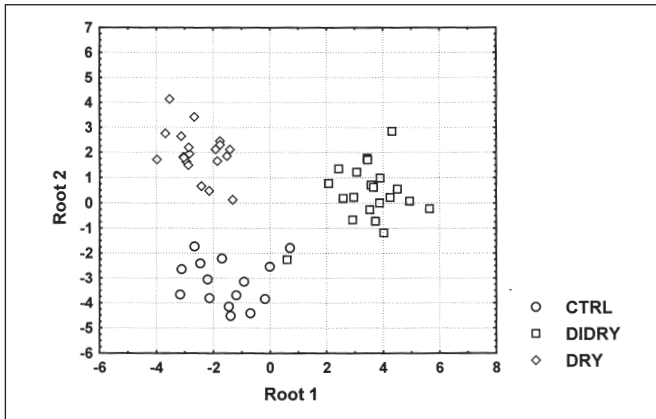
Fig. 3 - *The canonical square root was plotted against the first canonical root calculated by analysis of discriminance for the groups CTRL, DRY, and DIDRY at a initial diagnostic BST limit of 10 (i.e. all cases with BST values of 10 or more belong to the CTRL group). The graph shows the good separation between the groups.*

the groups, the better the analysis procedure discriminates between them. Figure 3 shows very good separation between the groups at BST 10.

To establish the extent to which the initial BST value influences the subsequent analysis of the HPLC runs, we repeated the whole calculation procedures using different initial BST limits, ranging between 5 and 15, meaning that for example at an initial BST limit of 8, all patients with BST 8 or more will belong to the CTRL group.

The difference between the mean canonical roots of each group was highest at a BST of approximately 10 (Fig. 4b). Figure 4a shows the distance to the CTRL group calculated by the analysis of discriminance plotted against the BST limit. For both groups, separation from the CTRL group is maximal near a BST limit of 10. Additionally we also found a good correlation between the BST value on which the initial diagnosis was based, and the distance to the CTRL group, calculated by analysis of discriminance ($r = -0.71$, $p<0.01$). Figure 5 shows the distance to the CTRL group and the BST value for each tear sample. There was no correlation between the distances to the CTRL group, the BST value, and the BUT value.

Multivariate analysis of discriminance is useful to classify HPLC tear protein patterns based on their similarity to clinically predefined groups (DRY, DIDRY, and CTRL): 98% of all samples were correctly classified.
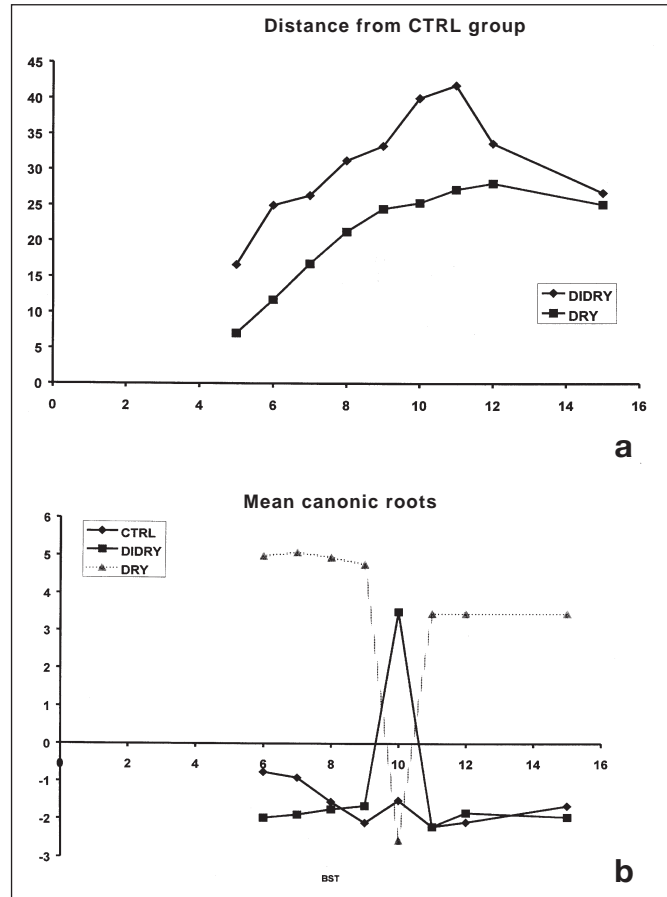

Fig. 4 - *Top (a): Correlation between the distances of the DIDRY and DRY groups from the BST limit the initial diagnosis is based on. Bottom (b): Means of the canonical roots calculated by analysis of discriminance of the CTRL, DRY, and DIDRY group were plotted against the BST value which is the upper limit for assignment to the DRY or DIDRY group.*
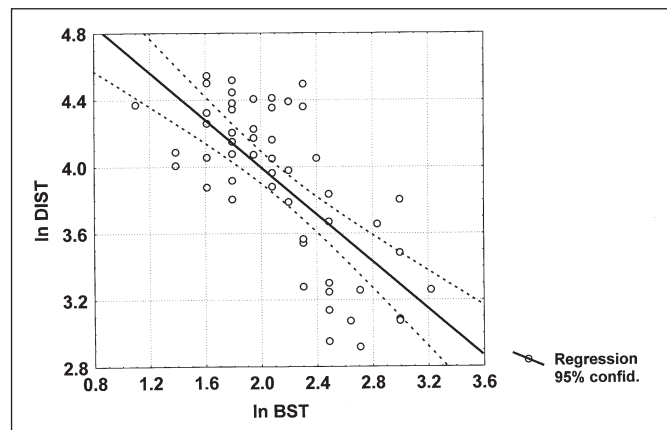

Fig. 5 - *Correlation between the distances of each sample from the CTRL group calculated by analysis of discriminance and the patient's BST value (r=-0.71, p<0.01).*

DISCUSSION

In the present study, tear protein patterns of non-diabetic and diabetic dry-eye patients were analyzed by size exclusion HPLC and compared to tears from healthy subjects. The HPLC data had a complex peak pattern but the main peaks (sIgA, lactoferrin, lysozyme, tear specific pre-albumin) could be easily detected and quantified. According to Boukes et al (29) and to a recent study by our group investigating the tear protein-patterns by SDS-PAGE (12), the concentration of most of these proteins was lower in the tears of dry-eye patients than in healthy subjects. However, in our previous study (12) none of these peak concentration differences became statistically significant. The present study using HPLC found significantly lower sIgA in dry-eye patients than controls. No significant differences in peak concentrations were observed between diabetic and nondiabetic dry-eye patients.

The tear protein patterns obtained by SDS-PAGE (12) showed a larger number of peaks/lane in patients suffering from dry-eye disease than in healthy subjects. This was significant for diabetic dry-eye patients. However, in the present study no significant difference in the number of peaks/HPLC run could be found. This may be because polyacrylamide gel electrophoresis of tear proteins offers the best resolution currently available (9). However, HPCL analysis of human tears is very fast and reliable. The overall SD in the retention time of one peak was only approximately 10 s. To overcome the effect of lactoferrin binding quantifiably to the HPLC column matrix, the pH and concentration of the buffer were varied (not shown here) and no significant difference was found in the HPLC pattern. Multivariate analysis of discriminance on the HPLC data of the three groups showed a significant difference ($p<0.01$) between them. This is because the analysis technique used relies not only on the main peaks and their parameters but on the whole complex pattern of the HPLC run. Although the present study found difference between the DIDRY and DRY groups none of the main peaks differed between these two groups. Thus, multivariate analysis taking into account the whole HPLC pattern appears to be more sensitive than the conventional comparison of a few single peaks. However, further studies are necessary on disease-associated disorders such as ex-ocrine dysfunction of the main lacrimal gland in diabetic patients (30).

Furthermore, analysis of discriminance classified patients as "dry-eye" or "not dry" correctly in a very high percentage (98%). This is slightly better than the classification based on analysis of SDS-PAGE tear protein patterns, where 92% were assigned correctly (12). Despite the known poor performance and reliability of the BST, the present study found a good correlation between BST and several parameters obtained by multivariate analysis. Changing the BST value that establishes whether a patient belongs to the dry-eye group or not, made the discriminance between groups less sharp. The groups were best discriminated at an initial BST value of 10, meaning that all patients with this value or higher are grouped as CTRL. Raising or lowering this limit reduces the quality of separation. Thus, changing the BST limit for the diagnosis of dry-eye to non realistic values raises the number of falsely-assigned patients in the initial group for analysis of discriminance. For example, setting the limit very low not only leads to the assignment of only the most severe cases to the DRY group, but also puts many patients with dry-eye in the CTRL group (false assignments). This will give a very "safe" and clear pattern for the severe dry-eye cases, but the pattern of the CTRL group will be distorted by the falsely-assigned cases. Thus, the overall differences between this DRY and CTRL group will be minimized compared to the differences found at a BST value of 10.

Significant differences were found between all groups. Analysis of HPLC runs of tears and subsequent statistical evaluation appear to be suitable for the detection of dry eyes with the HPLC method the analysis time is shorter than electrophoretic analysis, but the latter offers the advantage of higher resolution and can run several samples in parallel. HPLC analysis and the statistical routines used in this study can be automated, to provide a highly reliable procedure for the detection of dry eyes. Furthermore the HPLC method can improve the analysis of disease-associated tear proteins in clinical research.

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