HORIBA
Scientific

Application Note

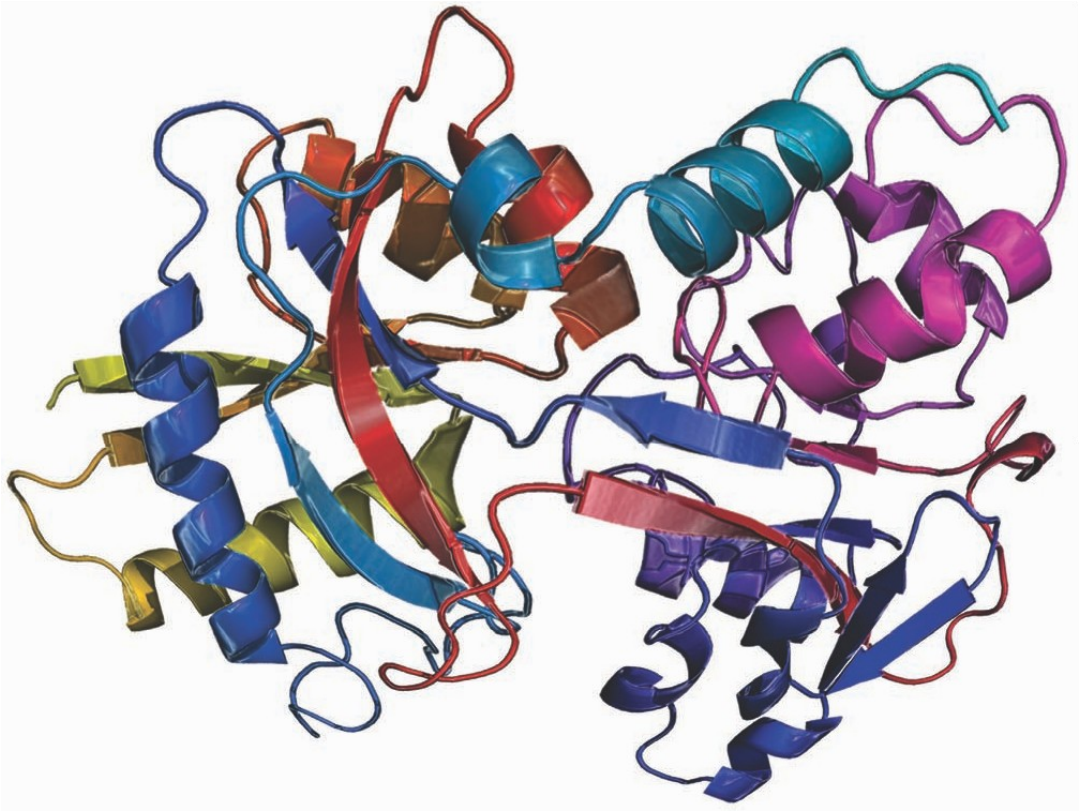
Life Sciences

Capillary Electrophoresis (CE)

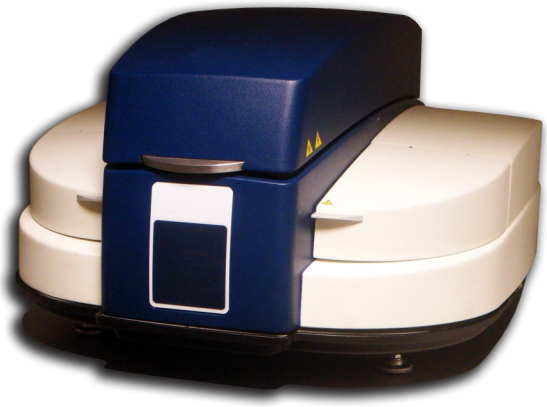
CEAN02

TRANSFERRIN ANALYSIS

By High Performance Capillary Electrophoresis



Analysis of Transferrin



Label free analysis using the HPCE-512

ABSTRACT

Transferrin isoforms including Carbohydrate Reduced Transferrin (CDT) were successfully analysed and resolved using the HPCE-512 High Performance Capillary Electrophoresis system. This demonstrates its ability to produce high quality repeatable data in terms of peak mobility (<0.5% RSD) and peak area (~2% RSD). Previous electrophoretic approaches to analyse these were highly complex and suffered from repeatability problems. Transferrin is a blood plasma glycoprotein involved in iron ion transport and is one of the conventional markers for chronic alcoholism.

This report demonstrates the capability of the HPCE-512 instrument for the label free analysis of Tf sialoforms using Capillary Zone Electrophoresis (CZE). Separation of native proteins using CZE is problematic due to protein adhesion to the inner surface of the capillary. This can lead to unstable currents, inefficient separations, poor reproducibility and sometimes a lack of analyte elution. Using the Successive Multiple Ionic Layer (SMIL) coating developed by Katayama et al 1998 [1] protein absorption was reduced and the Transferrin isoforms were separated based on differences in their net charge.

INTRODUCTION

Transferrin (Tf) is a major iron transporting protein in plasma. It is a glycoprotein and exhibits microheterogeneity in its amino-acid, iron and carbohydrate content. Tf has two asparagine linked N-glycan chains which are composed of neutral sugars forming di-, tri- and tetra antennary structures terminating with negatively charged sialic acid residues. The sialic acid content varies giving rise to isoforms with up to eight sialic acid residues. The molecular masses of these isoforms vary between 75 and 79 kDa and their isoelectric points range from 5.2 to 5.9. A normal profile is characterised by a distribution of sialoforms, with the pre-dominant form being tetrasialo-Transferrin. The di-, tri-, penta-, and hexasialo-forms are found at markedly lower levels.

Variation in serum sialoform content can be correlated to different pathological states. An increase in the relative levels of the lower sialoforms (i.e. disialo-Transferrin and asialo-Transferrin) has been associated with alcohol abuse. Carbohydrate deficient Transferrin (CDT) is the collective name for this group of less sialylated Transferrin isoforms.

MATERIALS AND METHODS

The Transferrin glycoform analyses were performed in a bare fused silica capillary of 62 cm effective length and 50 µm internal diameter. The SMIL capillary coating was generated using the protocol detailed by Katayama et al. except that the capillary was rinsed using a proprietary methodology. Electrophoresis was performed in borate buffer with a proprietary additive. The buffer was passed through a 0.2µm filter and degassed before use. The samples were injected at 5kV for 25 seconds and electrophoresed at a voltage of 16 kV. The samples were diluted in run buffer to a concentration of 1mg/ml with 5% ferric chloride solution. The sample was incubated for 10 minutes at room temperature before analysis to allow time for iron saturation.

The samples were desialylated by digestion with neuraminidase from Vibrio cholerae (BioChemika). Each 1mg/ml Tf sample was treated with a 1U/ml enzyme solution in a proprietary buffer. The samples were incubated at 37°C for 5 hours. Aliquots were removed every 60 minutes to assess the progress of the reaction. The digest was stopped by raising the pH to 8 and samples stored at 4°C. Between runs the capillary was flushed using a proprietary methodology after which an automated Ohm's Law Plot was performed.

RESULTS AND DISCUSSIONS

The rapid and efficient separation of the Transferrin glycoforms were analysed using both the Equiphase Vertexing Algorithm (EVA) and Generalised Separation Transform (GST) algorithm. GST is a method of combining the data from the 512 pixels in a natural way which preserves the peak shape information of the electropherograms while at the same time maximising the signal-to-noise ratio. A 10-fold increase in signal-to-noise using GST as compared to single electropherograms is typically observed. In addition, the absorbance A can be read directly off the GST plot peak height V as a function of time t (A = 0.434*V (t)).

EVA is an advanced pattern-recognition tool which maximizes the system resolution, and converts quantitative information to a peak height rather than area. In EVA the electropherograms are first analyzed to find local peaks. These are used first to perform vertexing (determine the point of origin of the bands) and then to produce a signal output. Thiourea was used to test the strength and stability of the electro-osmotic flow generated by the SMIL coating giving percentage RSDs over 40 runs for peak migration time, peak area and peak height were 0.7, 2.1 and 2.1 percent respectively. The GST and EVA processed data for the Holo-Transferrin sample is shown in **Figure 1** and the RSDs of tetra-sialo-Tf in **Table 1** and penta-sialo-Tf in **Table 2**.

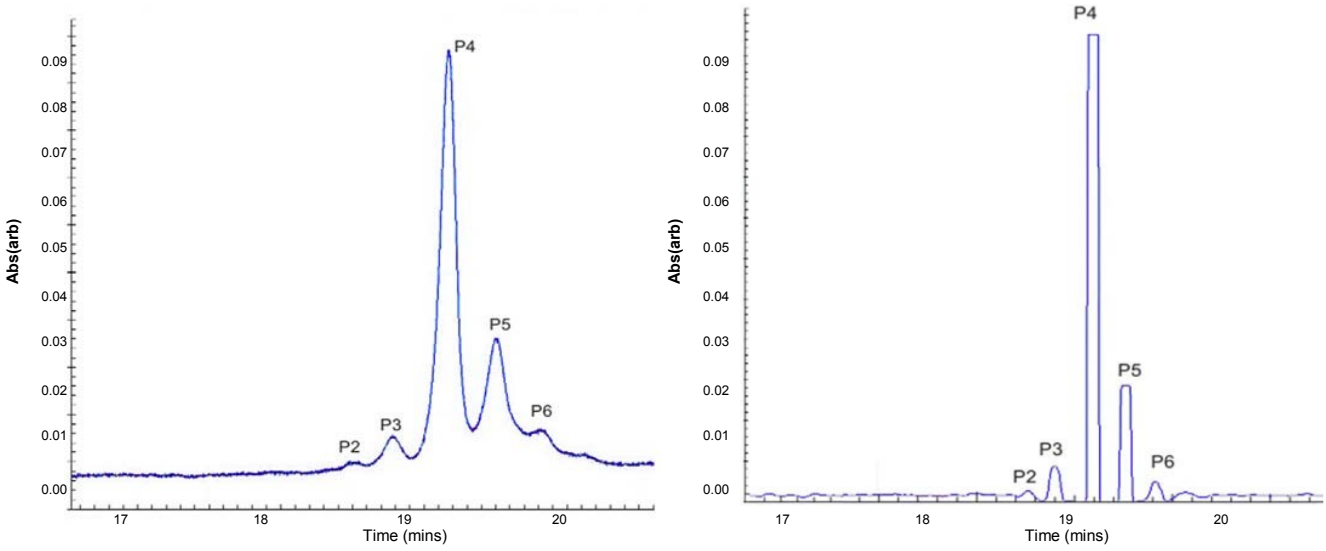


***Figure 1**: Panels A and B show GST electropherogram and EVA processed data for Human Holo-Transferrin. Peak identification: **P2** – disialo-Tf, **P3** – trisialo –Tf, **P4** – tetrasialo –Tf, **P5** – pentasialo –Tf and **P6** – hexasialo –Tf.*

	Mean	Standard deviation	%RSD
Peak time (mins)	15.35	0.057	0.37
Peak Area	0.0191	0.00039	2.04
Peak Height	0.000475	9.713E-06	2.04

***Table 1:** EVA repeatability data for peak migration time, peak area and peak height for tetra-sialoform Transferrin (**Tf-4**) (N=10).*

	Mean	Standard deviation	%RSD
Peak time (mins)	15.54	0.059	0.38
Peak Area	0.0048	7.78E-05	1.60
Peak Height	0.00012	1.91E-06	1.58

***Table 2**: EVA repeatability data for peak migration time, peak area and peak height for penta-sialoform Transferrin (**Tf-5**) (N=10).*

The stability and reproducibility of the coating and the HPCE instrument allows the chronological monitoring of the enzyme-based desialylation of Transferrin.

The neuraminidase treated Transferrin sample results are shown as EVA overlays between the hourly aliquots, to demonstrate the progress of the digest. The development of the reaction can be monitored by the appearance of desialylated isoforms and the decrease in peak area of the higher sialoforms.

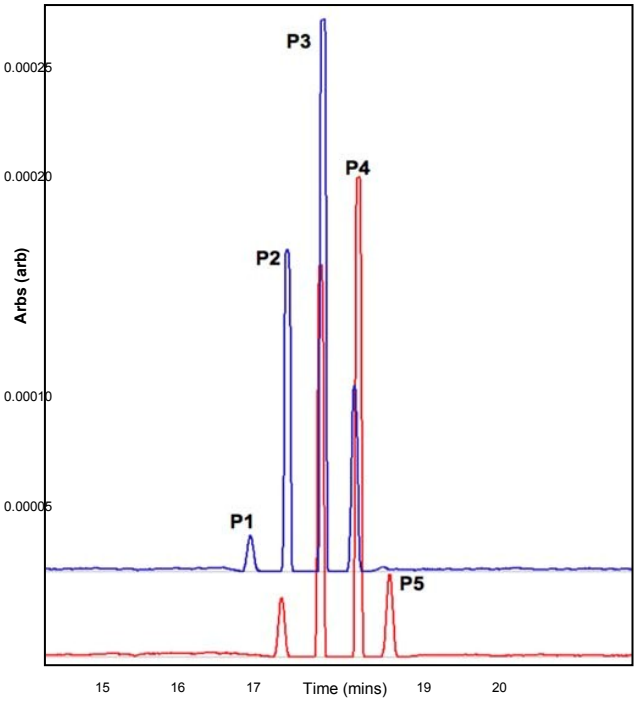


Figure 2: *Overlay of EVA processed data of Human Holo-Transferrin after treatment with neuraminidase for 1 hour (blue) and 2 hours (red). Peak identification: P1-monosialo-Tf, P2-disialo-Tf, P3-trisialo-Tf, P4- tetrasialo-Tf and P5-pentasialo-Tf*

At one hour (**Figure 2**) the major sialoform, Tetrasialo-Tf shows a marked decrease along with the pentasialo-Tf with an increase in tri and di-sialo-Transferrin and the appearance of the mono sialylated isoform. As the reaction developed progressive decrease in all the higher sialoforms can be seen (**Figures 3-4**).

After 5 hours (**Figure 5**) the major peaks present were the asialylated and monosialylated Transferrin isoforms. The shift in peaks from the 1 hour aliquot to the 5 hour aliquot is made obvious by the EVA data overlay (**Figure 6**).

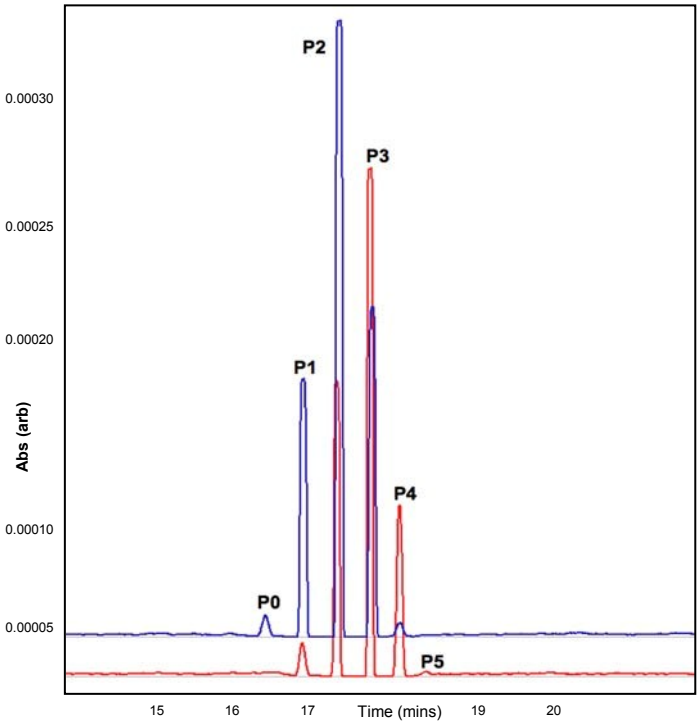


Figure 3*: Overlay of EVA processed data of Human Holo-Transferrin after treatment with neuraminidase for 2 hours (blue) and 3 hours (red). Peak identification: P0-asialo-Tf,P1-monosialo-Tf, P2-disialo-Tf, P3- trisialo-Tf and P4-tetrasialo-Tf.*

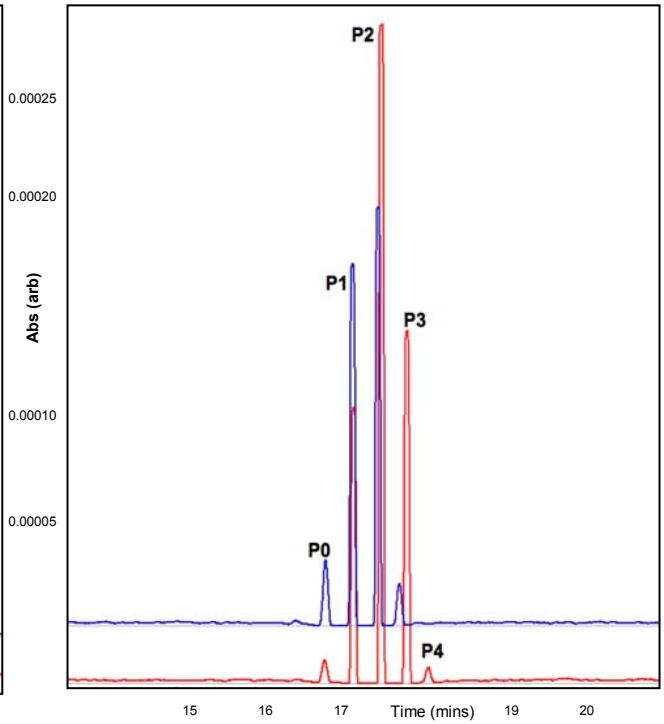


Figure 4*: Overlay of EVA processed data of Human Holo-Transferrin after treatment with neuraminidase for 3 hours (blue) and 4 hours (red). Peak identification: P0-asialo-Tf, P1-monosialo-Tf, P2-disialo-Tf, P3- trisialo-Tf and P4-tetrasialo-Tf.*

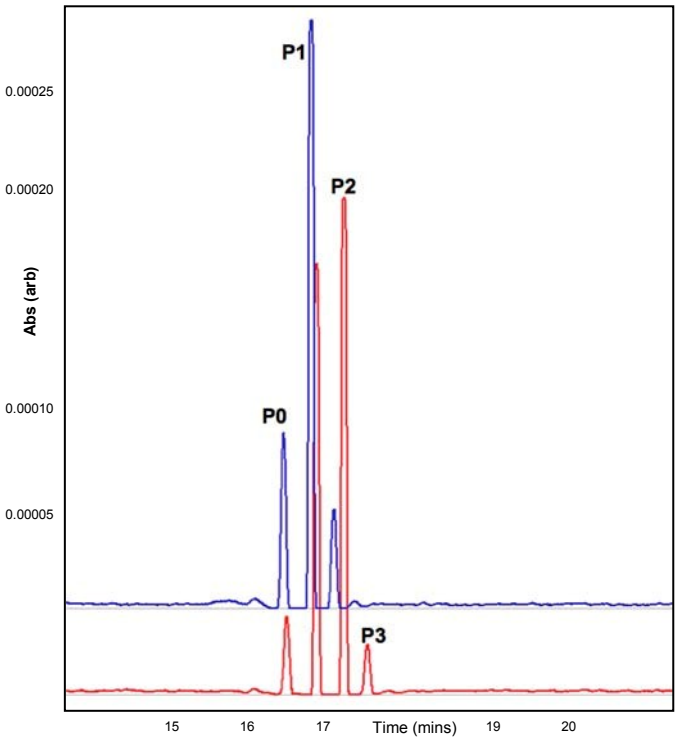


Figure 5: *Overlay of EVA processed data of Human Holo-Transferrin after treatment with neuraminidase for 4 hours (blue) and 5 hours (red). Peak identification: P0-asialo-Tf, P1-monosialo-Tf, P2-disialo-Tf and P3- trisialo-Tf.*

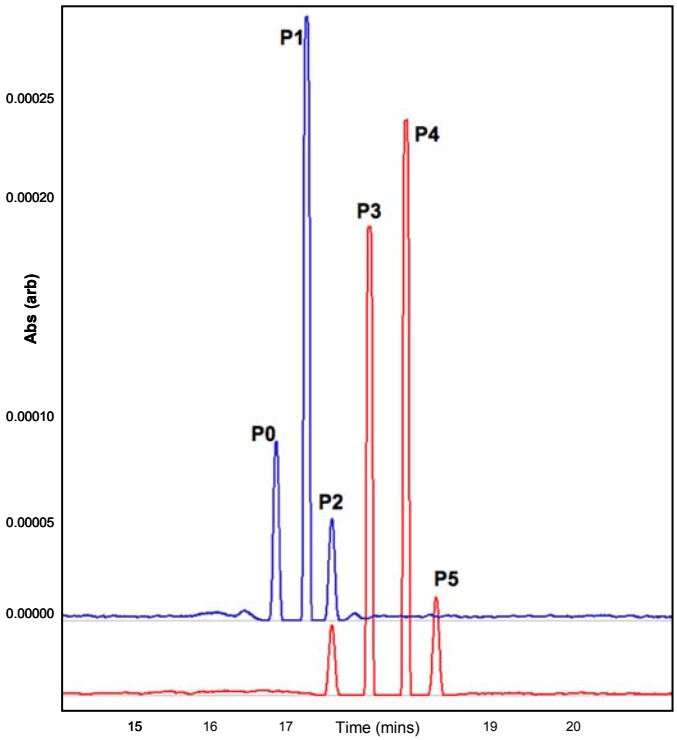


Figure 6*: Overlay of EVA processed data of Human Holo-Transferrin after treatment with neuraminidase for 1 hour (blue) and 5 hours (red). Peak identification: P0- asialo-Tf, P1-monosialo-Tf, P2-disialo-Tf, P3-trisialo-Tf, P4-tetrasialo-Tf and P5-pentasialo-Tf.*

CONCLUSION

There are several electrophoretic approaches that have been shown to be effective in the resolution of Transferrin isoforms, however most are difficult to set up and do not provide reproducible results.

The analysis of Transferrin isoforms on the HPCE-512 platform provides a new, reliable tool for the analysis of CDT isoforms with the aid of the SMIL coating.

The advantages offered by the HPCE-512 instrument in the analysis of Transferrin are simplicity of operation, speed of separation and the resolution obtained between the sialoforms. The robustness of the technique is demonstrated by the stability of the coating and the repeatability of the results obtained.

REFERENCE

H. Katayama, Y. Ishihama, N. Asakawa. 1998.
Stable Cationic Capillary Coating with Successive Multiple Ionic Polymer Layers for Capillary Electrophoresis.

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