*Clinical Chemistry* 46:4
483–492 (2000)

Enzymes and Protein Markers

# Investigation by Isoelectric Focusing of the Initial Carbohydrate-deficient Transferrin (CDT) and non-CDT Transferrin Isoform Fractionation Step Involved in Determination of CDT by the ChronAlcoI.D. Assay

Rolf Hackler,[1] Torsten Arndt,[2*] Angelika Helwig-Rolig,[3] Juergen Kropf,[3] Armin Steinmetz,[4] and Juergen R. Schaefer[1]

***Background:*** **The introduction of a new set of reagents for the determination of carbohydrate-deficient transferrin (CDT) as a marker of chronic alcohol abuse requires an independent evaluation of the analytic specificity of the test. This information is needed for correct interpretation and classification of test results.**

***Methods:*** **Isoelectric focusing on the PhastSystem™ followed by immunofixation, silver staining, and densitometry was used to validate the initial transferrin isoform fractionation step on anion-exchange microcolumns involved in the ChronAlcoI.D.™ assay.**

***Results:*** **The in vitro transferrin iron load was complete and stable. The CDT and non-CDT transferrin fractionation on anion-exchange microcolumns was reliable and reproducible (CV ≤10%). Except for quantitatively unimportant traces of trisialo-$Fe_2$-transferrin (<5% of total CDT), only asialo-, mono-, and disialo-$Fe_2$-transferrin were detected in the microcolumn eluates (n = 170). There was a loss of proportionally similar amounts of asialo-$Fe_2$-transferrin (during column rinsing) and disialo-$Fe_2$-transferrin (on the anion exchanger). Thus, the peak height ratios for disialo- and asialo-$Fe_2$-transferrin did not change from >1 (serum) to <1 (eluates) as described for the CDTect assays. The transferrin patterns in the ChronAlcoI.D. eluates were representative of those in serum. Transferrin D variants with isoelectric points close to that of trisialo-$Fe_2$-transferrin C1 did not cause overdetermination of CDT by the ChronAlcoI.D. test.**

***Conclusions:*** **The initial CDT and non-CDT fractionation step involved in determination of CDT by the ChronAlcoI.D. assay is efficient for eliminating non-CDT transferrins from serum before quantification of CDT in the final turbidimetric immunoassay. We recommend IEF for validation of other (commercial) CDT analysis methods and of odd CDT results.**

**© 2000 American Association for Clinical Chemistry**

Carbohydrate-deficient transferrin (CDT),[5] a group of transferrin isoforms lacking one or two N-glycans *(1–3)* and occurring with high prevalence in chronically increased alcohol consumption, is used for laboratory diagnosis of chronic alcohol abuse *(4, 5)*. Various CDT analytical methods have been published since the first description of CDT in 1976 *(6)*. Because CDT-specific chemical reactions or CDT antibodies are not currently available, CDT analysis requires a reliable separation of CDT from non-CDT transferrins. This can be done by electrophoretic or chromatographic procedures, e.g., isoelectric focusing (IEF) *(7, 8)*, capillary zone electrophoresis *(9)*, HPLC *(10, 11)*, or anion-exchange chromatography on microcolumns *(12)*. As we demonstrated previously *(8, 13)*, the CDT and non-CDT transferrin fractionation step is crucial for correct determination of

[1] Zentrum für Innere Medizin, Abteilung Kardiologie, and [3] Abteilung Klinische Chemie und Pathobiochemie-Zentrallaboratorium, Baldingerstrasse, Philipps-Universität, D-35033 Marburg, Germany.

[2] *bio*scientia, Institut für Laboruntersuchungen Ingelheim GmbH, Konrad-Adenauer-Strasse 17, D-55218 Ingelheim, Germany.

[4] Abteilung Innere Medizin und Zentrallabor, St. Nikolaus-Stiftshospital, D-56626 Andernach, Germany.

*Author for correspondence. Fax 049-6132-781-428; e-mail arndt@bioscientia.de.

Received November 30, 1999; accepted February 1, 2000.

[5] Nonstandard abbreviations: CDT, carbohydrate-deficient transferrin; IEF, isoelectric focusing; and CSF, cerebrospinal fluid.

CDT, regardless of which method is used. Detailed information on the reliability of the initial transferrin fractionation is needed for each CDT analytical method. This is true not only for routine analysis in accordance with good laboratory practice, but also when comparing values and diagnostic specificities and sensitivities of CDT obtained in different clinical settings with different CDT analytical methods *(13–15)*. Recently, a new CDT test, ChronAlcoI.D.™, was developed by Sangui BioTech, Inc. (Santa Ana, CA), formerly distributed by DPC/Biermann (Bad Nauheim, Germany) and currently by Biodiagnostics GmbH (Kiel, Germany). According to the test instructions, this assay measures asialo-, monosialo-, and disialo-$Fe_2$-transferrin and thus uses the common CDT definition given by Stibler *(16)*. Therefore, this test might be an interesting replacement for the widely used CDTect enzyme immunoassay, the production of which was terminated in January 1999. Despite its increased use for routine determination of CDT *(17)*, information about the reliability of the CDT and non-CDT fractionation step in the ChronAlcoI.D. assay is lacking at present. In continuation of our investigation of the CDTect assay *(13)*, we assessed this part of the ChronAlcoI.D. assay.

The aim of our study was to assess which transferrin isoforms are measured as CDT by the ChronAlcoI.D. assay. The following points, which are crucial for the specificity of the initial CDT and non-CDT transferrin isoform fractionation step, were investigated: (*a*) the efficiency of the in vitro transferrin iron-saturation step (which is used to establish a unique transferrin iron load by formation of $Fe_2$-transferrins and elimination of $Fe_1$- and $Fe_0$-transferrins); (*b*) the transferrin iron load stability during passage over the anion-exchange columns to prevent coelution of non-CDT and CDT transferrins with differing iron and sialic acid content but identical pI values; (*c*) the efficiency of the initial CDT and non-CDT transferrin fractionation step on the anion-exchange microcolumns; (*d*) the reproducibility of the anion-exchange microcolumn separation; and (*e*) possible effects of genetic transferrin D variants on the ChronAlcoI.D. assay results. Knowing which transferrin isoforms, and to what extent they are measured as CDT by the CDT analysis tests currently available, will undoubtedly be helpful for further studies on the pathomechanisms of CDT increases and for the diagnostic efficiency of CDT as the most specific marker of chronic alcohol abuse at present.

## Materials and Methods

### MATERIALS

All chemicals used for IEF and silver staining were of analytical grade and were obtained from Merck, except Pharmalytes 5–6 (Pharmacia/LKB), and acrylamide and bisacrylamide (Serva). Polyclonal rabbit IgG antibodies to human transferrin were from Dako. The ChronAlcoI.D. assay and the CDT control set (both from Sangui BioTech) were provided by DPC/Biermann.

### SERUM SAMPLES

All procedures were performed in accordance with the Helsinki Declaration of 1975, as revised in 1996.

Only surplus serum sample volumes from routine investigations were used. Blood was drawn after overnight fasting into sterile gel tubes (Sarstedt). The gel barrier consisted of a polymerized acrylic resin that does not affect the serum CDT concentration *(18)*. After clotting at room temperature for 30 min, the blood samples were centrifuged at 2000*g* for 10 min at 4 °C. Serum was removed immediately with disposable pipettes (to avoid contamination with microorganisms); aliquots were transferred into sterile, leak-proof plastic containers (Micro Tubes with screw cap; Sarstedt) and stored at −22 °C until the day of analysis.

### ChronAlcoI.D. ASSAY

Serum CDT concentrations and the CDT/transferrin ratios were determined by the ChronAlcoI.D. assay in accordance with the instructions of the manufacturer. The test comprises the following steps:

*In vitro transferrin iron saturation.* Serum or control sample (100 μL) and ferric saturation reagent (500 μL) are mixed and incubated for 5–10 min at room temperature.

*Anion-exchange microcolumn separation of CDT- and non-CDT-transferrins.* The $Fe^{3+}$-saturated serum or control sample (500 μL) is pipetted directly onto the top of the filter of the microcolumn. The sample drains until the top filter appears dry, and the effluent (load step) is discarded (non-CDT transferrins and CDT transferrins adsorb to the anion exchanger). The column is rinsed with 1.5 mL of elution buffer, and the effluent (rinse step) is discarded. An additional volume of 2.5 mL of elution buffer is pipetted onto the column filter, the buffer drains until the filter appears dry, and the eluate is collected in appropriately labeled test tubes (CDT transferrins are eluted).

*Preparation of the total transferrin solution.* The total transferrin solution is prepared while microcolumn separation is taking place. The $Fe^{3+}$-saturated serum or control sample (20 μL) is mixed with 800 μL of elution buffer. This sample is used in the following turbidimetric immunoassay for determination of the total serum transferrin concentration.

*Quantification of CDT and total transferrin by a microtiterplate turbidimetric immunoassay.* Calibrators, microcolumn eluates, and total transferrin dilution samples (200 μL of each) are pipetted directly into the bottom of each well, and atypical absorbance (background) is read at 405 nm (Dynatec MR 5000 reader; Dynex Technologies). Transferrin antibody solution (100 μL) is then added to each well. After gentle agitation for 15 min at room temperature, the absorbance is read at 405 nm. The analysis data

were evaluated with Dynex Revelation 3.2 software (Dynex Technologies). The results were reported as CDT/transferrin ratios as well as CDT concentrations.

Quality control for the whole ChronAlcoI.D. assay was in accordance with the guidelines of the German Federal Medical Association. Within each assay, serum pool aliquots with CDT values near the cutoff of 2.5–2.7% for women and men *(17)*, and two control samples with normal and increased CDT ratios (CDT control set; DPC/Biermann) were used for internal quality control. The control samples were placed at the beginning and the end of each set of samples. Control and serum samples were analyzed in duplicate. The laboratory regularly participates in external quality-control programs.

### IEF, immunofixation, and silver staining

The efficiency of the in vitro iron-saturation step, the transferrin iron load stability during passage through the microcolumns, the efficiency and reproducibility of the fractionation of CDT and non-CDT-transferrins, and the possible effects of transferrin D variants were assessed by IEF analysis of the transferrin isoform patterns in the corresponding sample aliquots after each intermediate step of the ChronAlcoI.D. CDT and non-CDT fractionation procedure. We investigated (*a*) the serum samples after $Fe^{3+}$-transferrin saturation (Fig. 1a, lanes A, and Fig. 3); (*b*) the column effluents after application of the iron-treated serum samples to the top of the anion-exchange microcolumns; (*c*) the column effluents after the columns were rinsed with 1.5 mL of elution buffer (Fig. 1a, lanes B); (*d*) the column eluates after the addition of 2.5 mL of elution buffer to the column (this eluate usually is used for quantifying CDT in the final turbidimetric immunoassay; Fig. 1a, lanes C; Fig. 2, lane 13; Figs. 3 and 4); and (*e*) additional 2 mol/L NaCl eluates that were used for recovery studies (usually not part of the original ChronAlcoI.D. test; Fig. 1a, lanes D).

Because of the limited capacity of the IEF system, it was impossible to analyze all samples immediately after the original ChronAlcoI.D. step. Thus, serum samples and column effluents and eluates were frozen immediately and stored at −22 °C until analysis (usually within 2 weeks). This sample storage (which is not part of the ChronAlcoI.D. assay, but which was part of our experimental setup) caused a partial loss of transferrin iron in the effluents and eluates, probably because of the ionic strength of the buffers used. To these samples, additional amounts of $Fe^{3+}$ were added, according to Hackler et al. *(8)*. Identical IEF transferrin patterns were obtained when we analyzed fresh samples and the same samples after freezing and additional $Fe^{3+}$ treatment in parallel. This demonstrates that our procedure is appropriate for readjusting the original complete transferrin iron load in thawed column effluents and eluates.

*IEF.* IEF was performed on the PhastSystem™, followed by immunofixation and silver staining as described by Hackler et al. *(8)* with the modifications described by Arndt et al. *(19)*. In short, polyacrylamide gels, pH 5–6 (43 × 50 × 0.45 mm; total acrylamide content, 5%; cross-linker content, 3%; Pharmalyte 5–6® diluted 1:16, by volume), adhered to a plastic support film (GelBond™ PAG film; Biozym-Diagnostik), were prepared in house and prefocused for 75 V-h. Using the Sample Applicator™ 8/1 (Pharmacia/LKB), we applied eight samples (1 μL of each sample). The sample applicator was inserted into the most cathodic position of the sample applicator arm. Sample application was performed for 15 V-h, and the separation was performed for 200 V-h.

*Immunofixation.* Immunofixation was done immediately after the IEF *(20)*. The gels were covered with 175 μL of polyclonal IgG antibodies to transferrin (50 μL of antibody diluted in 150 μL of 150 mmol/L NaCl) and incubated at room temperature in a moist chamber for 40 min. Unprecipitated (non-transferrin) proteins were removed by washing the gels with 150 mmol/L NaCl overnight with vigorous agitation.

*Silver staining.* Silver staining was carried out in the PhastSystem Development Unit™ according to Hackler and Kleine *(20)* with the following modification: The staining reaction was stopped by incubating the gel in 50 mmol/L EDTA (instead of 50 mL/L acetic acid). The gels were washed in deionized water for 2 h, dried in air, and kept for documentation. Transferrin bands were identified by parallel analysis of a cerebrospinal fluid (CSF) sample in each gel, showing (physiologically) asialo- to hexasialo-$Fe_2$-transferrin isoforms.

*Densitometry.* Densitometry was performed on a Preference densitometer (Sebia).

In adjusting the IEF sensitivity for detection of the CDT-transferrins, an overload of the tetrasialo-$Fe_2$-transferrin fraction was accepted. Thus, the intensity (Fig. 1a) and peak height or peak area (Fig. 1b) of this tetrasialo-$Fe_2$-transferrin fraction did not correlate with the transferrin content. In contrast to tetrasialo-$Fe_2$-transferrin, asialo-, mono-, di-, and trisialo-$Fe_2$-transferrin fractions were not overloaded. Thus, the peak heights or areas of these fractions could be used for determination of the percentage of the trisialo-$Fe_2$-transferrin contamination from total CDT.

### Other assays

To achieve comparable transferrin band and peak intensities between lanes A (serum) and D (2 mol/L NaCl eluate) of Fig. 1, the samples were diluted to a uniform total transferrin concentration of 8 mg/L. The total transferrin was determined with a Turbitimer and Turbiquant® transferrin reagent (Dade Behring).

## Results

We used IEF to evaluate the CDT and non-CDT transferrin fractionation step involved in determination of CDT by the ChronAlcoI.D. assay. A total of 170 column eluates were analyzed. Of these, 62 eluates (Fig. 1a, lanes C) were investigated together with the corresponding $Fe^{3+}$-treated serum samples, and 36 were investigated together with the corresponding serum samples (Fig. 1a, lanes A), column effluents (rinse step; Fig. 1a, lanes B), and 2 mol/L NaCl eluates (Fig. 1a, lanes D); for the nomenclature, see *Materials and Methods*. As the main result of our study, we found only CDT transferrin isoforms (asialo-, monosialo-, and disialo-$Fe_2$-transferrin) in the ChronAlcoI.D. microcolumn eluates, except for quantitatively unimportant traces (<5% of total CDT) of trisialo-$Fe_2$-transferrin. In testing the different points discussed earlier, we obtained the following results.

### in vitro transferrin iron saturation

When in vitro transferrin iron saturation is complete, only $Fe_2$-transferrin bands, but not $Fe_0$- and $Fe_1$-transferrin bands, should appear in the transferrin IEF band pattern. Furthermore, the transferrin band pattern should be unaffected by an additional (second) $Fe^{3+}$-transferrin saturation step. We tested 98 serum samples. Typical transferrin isoform band patterns obtained by IEF after $Fe^{3+}$-transferrin saturation are shown in lanes A of Fig. 1a and lane " Serum" of Fig. 3. $Fe_2$-transferrin bands, but not $Fe_0$- and $Fe_1$-transferrin bands, were detected. The bands corresponded to di-, tri-, tetra-, penta-, and hexasialo-$Fe_2$-transferrin (from cathode to anode) as verified by comparison with lane "CSF" in Figs. 1a and 3. The more intense bands of disialo-$Fe_2$-transferrin and additional bands of monosialo- and asialo-$Fe_2$-transferrins seen in lanes A of Fig. 1a from the alcoholics with homozygous transferrin C1 [alcoholic (Tf C1)] and heterozygous transferrin C1D [alcoholic (Tf C1D)] phenotypes in comparison with lane A of the control [control (Tf C1)] are attributable to the chronic alcohol abuse of these patients. The complex transferrin isoform band patterns in Fig. 1a, lanes A–D of the alcoholic with transferrin C1D [alcoholic (Tf C1D)] are explained further below.

The additional amounts of $Fe^{3+}$ added to the samples according to the method of Hackler et al. *(8)* did not affect either the number of transferrin bands or the positions of the transferrin bands within the gel. From this it follows that the ChronAlcoI.D. $Fe^{3+}$-transferrin saturation step is effective in achieving a uniform transferrin iron load by complete elimination of $Fe_0$- and $Fe_1$-transferrins.

### transferrin iron load stability during passage over the anion-exchange microcolumn

Transferrin iron loss, and thus reformation of $Fe_0$- and $Fe_1$-transferrins, can occur under nonoptimal pH conditions during the analytical process *(8)*. This loss can cause distinct overdetermination of CDT because of coelution of transferrin isoforms with differing sialic acid and iron content but the same pI *(8)*. Thus, we checked the 2.5-mL eluates (which are used in the final CDT quantification step of the ChronAlcoI.D. assay) for the presence of $Fe_0$- and $Fe_1$-transferrins immediately after the elution step and after additional amounts of $Fe^{3+}$ were added according to the method of Hackler et al. *(8)*. Altogether, 170 column eluates (which are used in the final turbidimetric immunoassay involved in the ChronAlcoI.D. test) were assessed (lanes C of Fig. 1a; lane 13 of Fig. 2; lanes "Eluate" of Fig. 3; and lanes 1–7 of Fig. 4). When fresh effluents and eluates (as obtained by the original ChronAlcoI.D. procedure) were analyzed, $Fe_0$- and $Fe_1$-transferrins were not detected. The added $Fe^{3+}$ did not affect the transferrin band patterns, which demonstrates a complete and stable transferrin iron load during passage over the microcolumn. Examination of the column effluents and eluates only a few hours after the elution step showed a small transferrin iron loss (weak bands of $Fe_0$- and $Fe_1$-transferrins). The loss was marked after storage of the eluates for 3 months at −22 °C. After we added $Fe^{3+}$ to these samples (to readjust the original transferrin iron load), we could no longer detect bands of $Fe_0$- and $Fe_1$-transferrin or of more highly sialylated non-CDT transferrins by IEF. This demonstrates that the transferrin iron loss occurred during the sample storage, but not during the CDT and non-CDT fractionation on the anion-exchange microcolumns. To test this point further, we determined the CDT/transferrin ratio and the CDT concentration from column eluates of seven serum samples with normal and increased CDT after storage for 1, 2, 3, and 6 days at 4–8 °C without adding further amounts of $Fe^{3+}$. The corresponding CVs were between 3.2% and 10%, which is close to the imprecision of the ChronAlcoI.D. assay reported by Arndt et al. *(17)*. We conclude that the transferrin iron saturation in accordance with the test instructions of the ChronAlcoI.D. assay was complete and stable during the CDT and non-CDT transferrin fractionation procedure. It permits reliable elimination of $Fe_0$- and $Fe_1$-transferrins and thus a uniform $Fe_2$-transferrin iron load in serum samples. The column eluates can be stored at 4–8 °C overnight without the risk of false CDT results in the final ChronAlcoI.D. turbidimetric immunoassay.

### CDT and non-CDT transferrin fractionation

This part of the assay consists of three intermediate steps: application of the $Fe^{3+}$-treated serum to the microcolumn, rinsing the column, and eluting the CDT transferrins. The specificity of the microcolumn separation was tested by IEF of the transferrin isoforms occurring in these matrices. We analyzed 6 column effluents from the loading step, 36 column effluents from the washing step, and 170 column eluates. We did not detect transferrins in the column effluent from the loading step, confirming that there was complete adsorption of all transferrin isoforms to the anion exchanger (not shown). Distinct amounts of asialo-$Fe_2$-transferrin appeared in all column effluents during

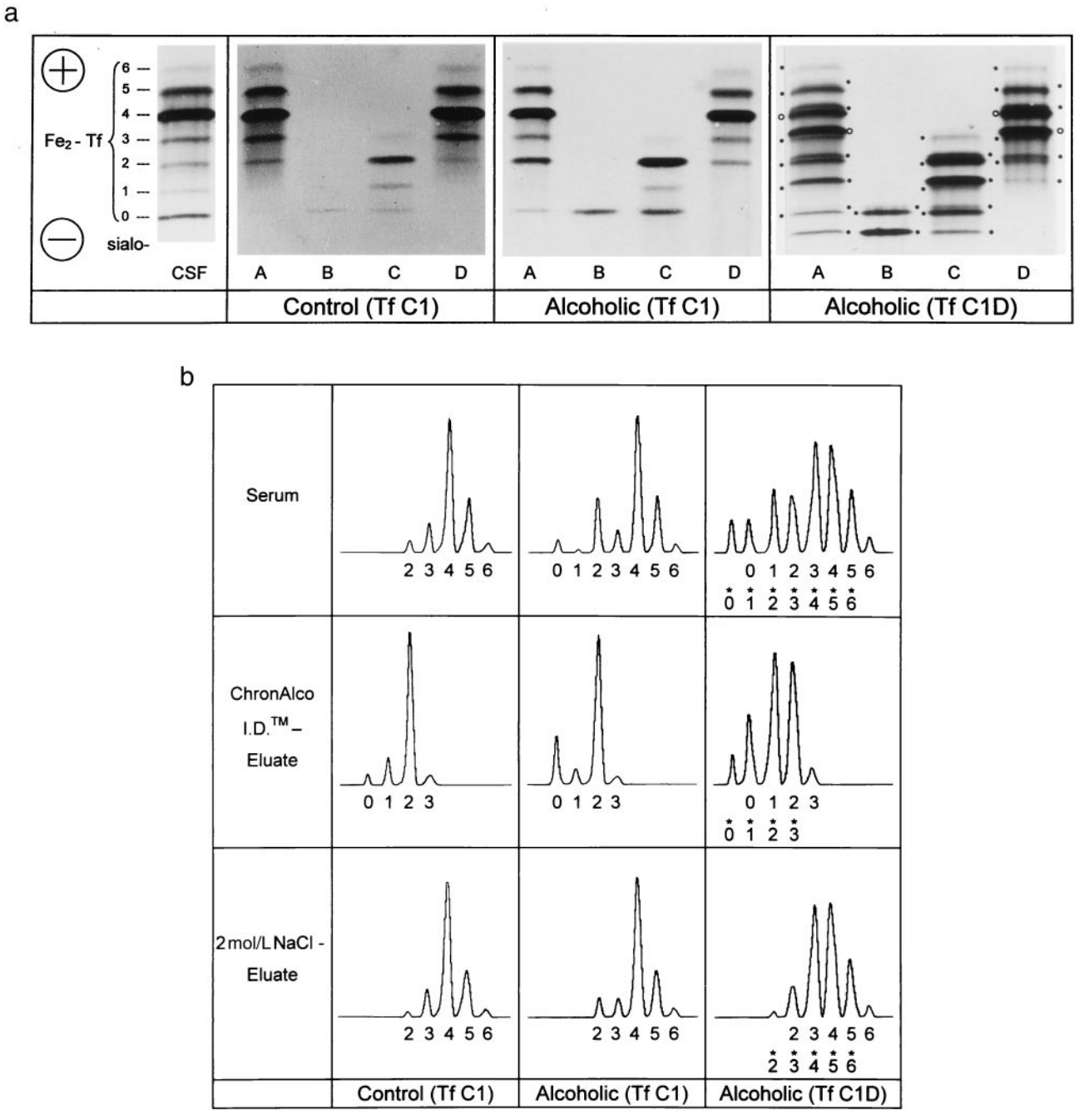


Fig. 1. Efficiency of the non-CDT and CDT fractionation involved in the determination of CDT by the ChronAlcoI.D. assay evaluated by IEF (*a*) and IEF/densitometry (*b*).

(*a*), *lanes A*, serum from a healthy control with transferrin C1 phenotype [*Control (Tf C1)*; 2.3% CDT; 87 mg/L CDT], an alcoholic with transferrin C1 phenotype [*Alcoholic (Tf C1)*; 8.8% CDT; 258 mg/L CDT], and an alcoholic with transferrin C1D phenotype [*Alcoholic (Tf C1D)*; 12% CDT; 399 mg/L CDT] after in vitro transferrin iron saturation. *Lanes B*, corresponding column effluents after the rinse step; *lanes C*, corresponding original anion-exchange microcolumn eluates; *lanes D*, corresponding anion-exchange microcolumn 2 mol/L NaCl eluates (no ChronAlcoI.D. step; generated for recovery studies). *Lane CSF*, transferrin isoform band pattern of CSF after in vitro transferrin iron saturation used for identification of the transferrin bands in *lanes A–D*. Numbers to the *left* of *lane CSF* indicate number of sialic acid residues. The fact that only $Fe_2$-transferrins (and no $Fe_1$- and $Fe_0$-transferrins) are visible in *lanes A–D* indicates a complete and stable in vitro transferrin iron load. Aside from traces of trisialo-$Fe_2$-transferrin, we did not find more highly sialylated non-CDT-transferrins (especially tetrasialo-$Fe_2$-transferrin) in the original eluates (*lanes C*). This demonstrates a reliable retention of these transferrins on the anion exchanger. The presence of disialo-$Fe_2$-transferrin in the 2 mol/L NaCl eluates (*lanes D*) reflects the partial retention of this transferrin isoform on the anion-exchange column. The complex transferrin band patterns in *lanes A–D* of the alcoholic with transferrin C1D [*Alcoholic (Tf C1D)*] are explained in *Results*. (*b*), *asterisks* above the number of sialic acid residues indicate the peaks of transferrin D isoforms. Because of various dilutions, comparison of the intensities or peak heights of different transferrin isoform fractions is possible only within the same matrix and between serum and NaCl eluate (*lanes A* and *D* in *a* and densitograms in *b*), which were diluted to a uniform total transferrin concentration of 8 mg/L. *Tf*, transferrin.

the wash step, regardless of whether normal or increased amounts of CDT appeared in the serum sample (Fig. 1a, lanes B; Fig. 2, lanes 5 and 6), revealing that this CDT transferrin was partially lost when the microcolumns were rinsed. The column eluates (which are used for the final CDT quantification) contained asialo-, mono-, and disialo-$Fe_2$-transferrin, which collectively are referred to as CDT *(16)*, and traces of trisialo-$Fe_2$-transferrin (Fig. 1a, lanes C; Fig. 2, lane 13; Fig. 3, lane "Eluate"; Fig. 4). The recovery of CDT was tested by eluting the microcolumns with 1 mL of 2 mol/L NaCl (the optimal volume for complete elution of all transferrin isoforms from the anion exchanger was assessed by fractionated elution in 500-$\mu$L steps). The 2 mol/L NaCl eluates contained mainly more highly sialylated, non-CDT transferrins (tri-, tetra-, penta-, and hexasialo-$Fe_2$-transferrin) but also disialo-$Fe_2$-transferrin. The latter finding reflects a partial retention of this CDT transferrin by the anion exchanger. Altogether, we found a partial loss of asialo-$Fe_2$-transferrin in the column rinse step and a partial loss of disialo-$Fe_2$-transferrin on the anion exchanger. This incomplete recovery of CDT was observed for serum samples with normal as well as with increased CDT concentration.

We tested by fractionated rinsing and fractionated elution whether the elution buffer volumes for rinsing the microcolumns and eluting the CDT transferrins, and thus the CDT recovery, could be optimized (Fig. 2). As shown in Fig. 2, an elution buffer volume of 1.0 mL (instead of 1.5 mL) would be sufficient for rinsing the column. With this volume, asialo-$Fe_2$-transferrin would be completely retained on the anion exchanger (Fig. 2, lanes 1–4). Rinse volumes >1.0 mL, e.g., 1.5 mL in accordance with the test instructions, cause a partial loss of asialo-$Fe_2$-transferrin (Fig. 2, lanes 5 and 6). Increasing the buffer volume for eluting the CDT transferrins from 2.5 mL (original volume) to 3.0 mL (Fig. 2, lane 12) would improve the recovery of disialo-$Fe_2$-transferrin, but at the same time it would exacerbate the coelution of trisialo-$Fe_2$-transferrin. Reducing the elution buffer volume from 2.5 mL to 2.0 mL allows almost complete retention of trisialo-$Fe_2$-transferrin (Fig. 2, lane 10), but it also allows higher loss of disialo-$Fe_2$-transferrin on the anion exchanger.

To assess the extent of CDT loss by the ChronAlcoI.D. transferrin isoform fractionation step, we diluted serum samples and the corresponding microcolumn eluates (which are used in the final turbidimetric immunoassay) to obtain similar peak heights for disialo-$Fe_2$-transferrin (Fig. 3). Taking into account the different dilution factors, the CDT loss was estimated to be ~30%. We also used these samples to test whether the CDT transferrin patterns in the eluate are representative of those in the iron-treated serum samples. Comparing the serum disialo-$Fe_2$-transferrin/asialo-$Fe_2$-transferrin peak height ratios with those of the corresponding ChronAlcoI.D. eluate (Fig. 3) showed that the transferrin isoform patterns in the ChronAlcoI.D. column eluates are representative of the transferrin isoform pattern in the serum sample. The almost identical disialo-$Fe_2$-transferrin/asialo-$Fe_2$-transferrin peak height ratios in serum (3.2) and eluate (3.4) indicate a proportionally similar loss of asialo-$Fe_2$-transferrin (during column rinsing) and disialo-$Fe_2$-transferrin (on the anion exchanger). Thus, the disialo-$Fe_2$-transferrin/asialo-$Fe_2$-transferrin peak height ratios did not change from >1 (serum) to <1 (eluate) as described for the CDTect assays *(13)*. Identical results were obtained for serum samples with normal and increased CDT concentration (Figs. 1 and 3). An important fact is that increased CDT fractions in serum were always reflected in the corresponding microcolumn eluates. This is another indication that the ChronAlcoI.D. microcolumn eluates are representative of the serum sample. Altogether, the original ChronAlcoI.D. CDT and non-CDT transferrin fractionation procedure

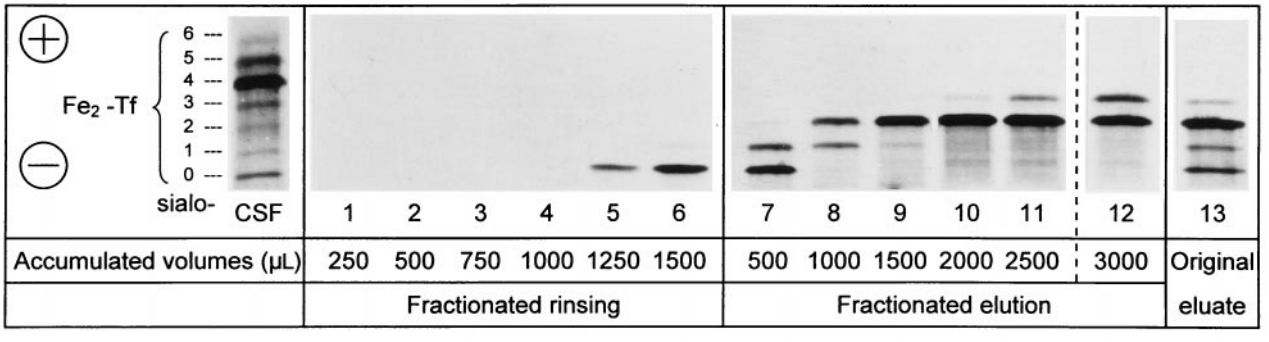


Fig. 2. Efficiency of the elution buffer volumes for rinsing the anion-exchange microcolumns after application of the serum samples to the column and for eluting the CDT-transferrins from the anion exchanger.

*Lanes 1–6*, transferrin isoform patterns after fractionated rinsing in six steps (250 $\mu$L of elution buffer in each step) instead of application of the original 1500-$\mu$L volume in one step to the column. *Lanes 7–11*, transferrin isoform patterns after fractionated elution in five steps (500 $\mu$L of elution buffer in each step) instead of application of the original 2.5-mL volume in one step. *Lane 12*, IEF transferrin band pattern after the accumulated elution volume was increased to 3.0 mL. *Lane 13*, transferrin pattern in the original ChronAlcoI.D. eluate, which was obtained in accordance with the test instructions. The original buffer volume for rinsing the column causes a partial loss of asialo-$Fe_2$-transferrin. Increasing the buffer volume for the CDT-transferrin elution step yields a distinct coelution of trisialo-$Fe_2$-transferrin. Similar results were obtained for serum samples with normal CDT/transferrin ratios and normal CDT concentrations. The CDT/transferrin ratio of the serum shown here was 9.0%, and the CDT concentration was 369 mg/L. Because of the different effective elution volumes (250 $\mu$L for each of *lanes 1–6*; 500 $\mu$L for each of *lanes 7–12*), the intensities of the transferrin isoform fractions in *lanes 1–12* are not comparable with those in *lane 13* (effective elution volume, 2500 $\mu$L). Numbers to the *left* of *lane CSF* indicate number of sialic acid residues. *Tf*, transferrin.

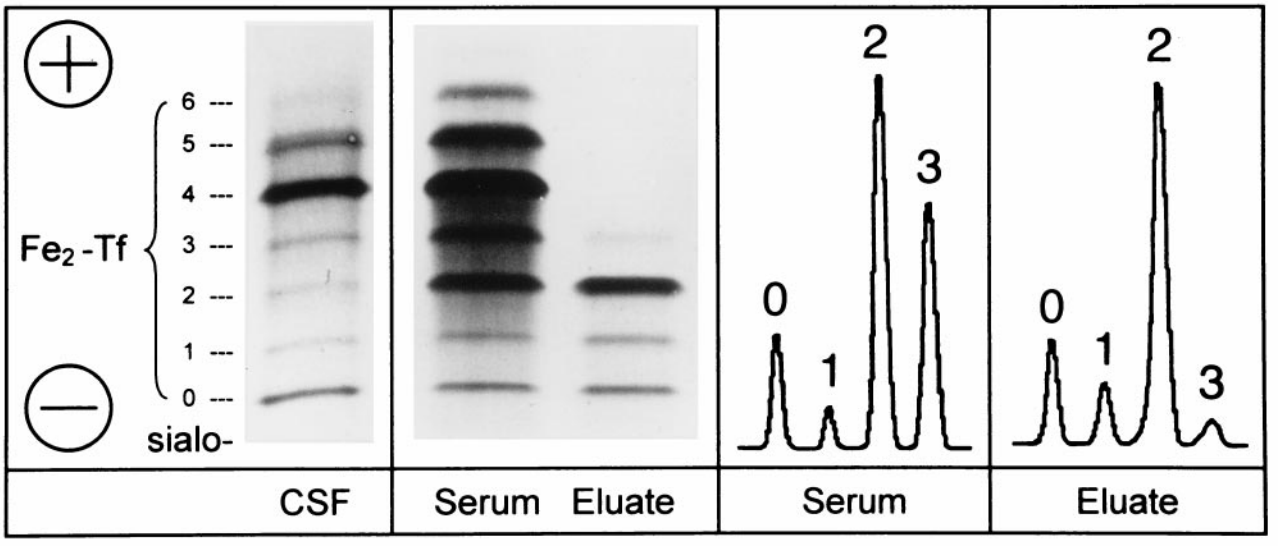


Fig. 3. Transferrin isoform pattern in serum after transferrin $Fe^{3+}$-saturation and after microcolumn CDT and non-CDT fractionation.

Serum and eluate were diluted to obtain similar peak intensities for disialo-$Fe_2$-transferrin. In comparing the different dilution factors, the CDT loss on the anion exchanger was estimated to be ~30%. Almost identical peak height ratios of disialo- to asialo-$Fe_2$-transferrin indicate that the losses of asialo-$Fe_2$-transferrin (in the column rinse step) and of disialo-$Fe_2$-transferrin (on the anion exchanger) are proportionally comparable. Numbers to the *left* of *lane CSF* indicate number of sialic acid residues. The corresponding densitograms [*right-hand panels*; only asialo- to trisialo-$Fe_2$-transferrin bands (*peaks 0–3*) were scanned] clearly show that the CDT transferrin pattern in the ChronAlcoI.D. eluate is representative for that in the serum. The CDT/transferrin ratio of the serum shown here was 7.5%, and the CDT concentration was 330 mg/L. *Tf*, transferrin

yields reliable separation and thus specific determination of CDT in the final turbidimetric immunoassay. The elution buffer volume of 2.5 mL seems to be a good compromise between maximum analytical specificity (exclusion of more highly sialylated non-CDT transferrins, e.g., trisialotransferrin and, most important, tetrasialotransferrin) on the one hand and maximum recovery of CDT on the other hand.

### REPRODUCIBILITY OF THE MICROCOLUMN PERFORMANCE

The within-run reproducibility of the whole CDT and non-CDT transferrin fractionation step was tested by processing seven aliquots of the same serum sample with increased CDT using seven different columns in one analytical run. When we calculated the means, SDs, and CVs of the peak height ratios of disialo-$Fe_2$-transferrin/monosialo-$Fe_2$-transferrin, disialo-$Fe_2$-transferrin/asialo-$Fe_2$-transferrin, and monosialo-$Fe_2$-transferrin/asialo-$Fe_2$-transferrin for each serum sample, we obtained imprecision values (CVs) of 5%, 9%, and 10%, respectively (Fig. 4), which were comparable to the intraassay imprecision (9%) of the whole ChronAlcoI.D. *(17)*.

### EFFECTS OF TRANSFERRIN D VARIANTS ON THE DETERMINATION OF CDT BY THE ChronAlcoI.D. ASSAY

Transferrin D variants have been reported to interfere with the determination of CDT, producing false positives with respect to chronic alcohol abuse *(7)*. We analyzed serum samples of an alcoholic and a healthy proband with heterozygous transferrin D phenotypes. The genetic transferrin D variants had isoelectric points very close to that of trisialo-$Fe_2$-transferrin C1. The transferrin isoform band patterns in serum, effluent (rinse step), eluate, and NaCl eluate of the alcoholic are shown in Fig. 1a [alcoholic (Tf C1D)]. Because both transferrin C1 and transferrin D also appear as transferrin isoforms with different sialic acid contents, the IEF transferrin isoform band patterns were complex. The transferrin bands belonging to transferrin C1 are indicated by dots to the left and those of transferrin D variant are indicated by dots to the right of the bands [Fig. 1a, alcoholic (Tf C1D)]. The open circles in lanes A and D of the alcoholic (Tf C1D) pattern indicate the tetrasialo-$Fe_2$-transferrin fractions of transferrin C1 (open circle to the left of the band) and transferrin D (open circle to the right of the band). The transferrin isoform band pattern in serum after in vitro transferrin $Fe^{3+}$ saturation is shown in lane A. Distinct bands of disialo-$Fe_2$-transferrin and additional bands of mono- and asialo-$Fe_2$-transferrin in comparison with lane A of the control (Fig. 1a) reflect the chronic alcohol abuse of this patient.

The IEF transferrin band pattern in the column effluent (rinse step) is shown in lane B of the alcoholic (Tf C1D) pattern in Fig. 1a. The two bands represent asialo-$Fe_2$-transferrin D (most cathodic and more intense band) and asialo-$Fe_2$-transferrin C1 (more anodic band). Lane B shows that larger amounts of the asialo-$Fe_2$-transferrin D in comparison with asialo-$Fe_2$-transferrin C1 were lost when the column was rinsed. Lane C of the alcoholic (Tf C1D) pattern in Fig. 1a shows the transferrin isoform pattern in the column eluate (CDT elution step). The band pattern is complex, with two distinct bands corresponding to disialo-$Fe_2$-transferrin C1 (more anodic band) and disialo-$Fe_2$-transferrin D (more cathodic band). The first

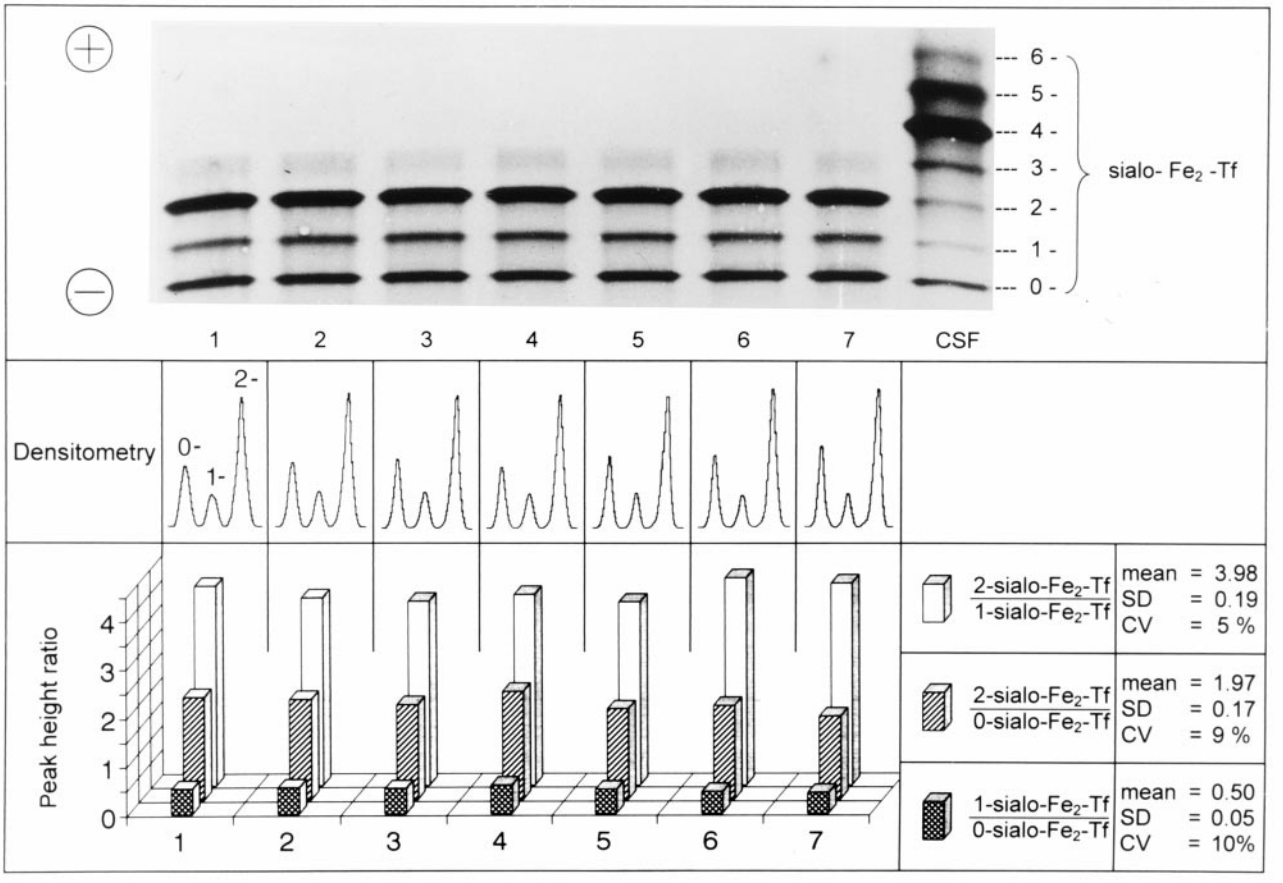


Fig. 4. Reproducibility of the ChronAlcoI.D. anion-exchange microcolumn performance.

*Lanes 1–7*, processing of seven different aliquots from seven different microcolumns of the same serum sample with an increased CDT concentration in accordance with the ChronAlcoI.D. procedure, followed by IEF/densitometry of the microcolumn eluates. Except for quantitatively unimportant traces of trisialo-$Fe_2$-transferrin (<5% of total CDT), only bands of CDT transferrins [asialo- (*0-sialo-$Fe_2$-Tf*), monosialo- (*1-sialo-$Fe_2$-Tf*), and disialo-$Fe_2$-transferrin (*2-sialo-$Fe_2$-Tf*)] are visible. The means, SDs, and CVs of the peak height ratios of *lanes 1–7* are given. An imprecision of ≤10% indicates very uniform microcolumn performance [CV of the whole ChronAlcoI.D. assay, 9% *(17)*]. The CDT/transferrin ratio of the serum sample shown here was 12.4% ± 0.8%, and the CDT concentration was 463 ± 6 mg/L (mean of seven analyses ± SD). Numbers to the *right* of *lane CSF* indicate number of sialic acid residues.

overlaps with traces of trisialo-$Fe_2$-transferrin D, the latter with monosialo-$Fe_2$-transferrin C1. There are also traces of trisialo-$Fe_2$-transferrin C1 (most anodic band), monosialo-$Fe_2$-transferrin D, asialo-$Fe_2$-transferrin C1, and asialo-$Fe_2$-transferrin D (from anode to cathode). For determination of CDT from this sample, it is important that tetrasialo-$Fe_2$-transferrin of the transferrin D variant does not appear in the column eluate (Fig. 1a, alcoholic (Tf C1D), lane C). Coelution of trisialo-$Fe_2$-transferrin D in the column eluate might cause an overdetermination of CDT. However, the loss of larger amounts of asialo-$Fe_2$-transferrin D during column rinsing [Fig. 1a, lane B of the alcoholic (Tf C1D) pattern] compensates partially for this lack of specificity. Indeed, we determined a CDT/transferrin ratio of 2.3% and a CDT concentration of 83 mg/L for the healthy proband with the transferrin C1D phenotype. Both values were below the corresponding cutoffs of 2.5–2.7% and 100–110 mg/L *(17)*. For the alcoholic, we measured a CDT/transferrin ratio of 12% and a CDT concentration of 399 mg/L. Altogether, the individual alcohol consumption of these two persons was correctly reflected by the ChronAlcoI.D. results (despite the presence of transferrin D variants).

## Discussion

The specific chromatographic or electrophoretic separation of non-CDT and CDT transferrins is complicated by the identical isoelectric points of transferrin isoforms with differing iron and sialic acid content, e.g., disialo-$Fe_2$-transferrin as a major CDT transferrin and tetrasialo-$Fe_1$-transferrin as the main non-CDT transferrin *(8, 21–23)*. In establishing a uniform transferrin iron load, the number of potential serum transferrin isoforms is reduced from 36 to 9 for homozygous and from 72 to 18 for heterozygous transferrin phenotypes *(21)*. Minimal isoelectric point differences of 0.1 pH units for transferrins with a uniform iron load but different sialic acid content, e.g., disialo- and trisialo-$Fe_2$-transferrin *(21)*, further complicate CDT analysis. Therefore, a complete and stable transferrin iron load and reliable CDT and non-CDT fractionation are prereq-

uisites for correct determination of CDT *(8, 13)*. We investigated the initial CDT and non-CDT transferrin fractionation step used in the ChronAlcoI.D. assay. The data obtained in this study show reliable separation of CDT and non-CDT transferrins on the anion-exchange microcolumns and a high precision in the microcolumn performance. Whether trisialo-$Fe_2$-transferrin should be (partially) incorporated in CDT has been discussed previously *(13, 24)*. Recently, Lipkowski et al. *(25)* reported that including trisialo-$Fe_2$-transferrin in CDT does not improve the diagnostic performance of CDT as the most specific marker of chronic alcohol abuse at present. It should be taken into account that "trisialo-tests" are potentially more strongly affected by transferrin D variants than tests that do not incorporate this transferrin isoform in CDT. When CDT tests incorporating (partially) trisialo-$Fe_2$-transferrin in CDT (trisialo-tests) are used, the coelution of tetrasialo-$Fe_2$-transferrin D with trisialo-$Fe_2$-transferrin C1 might cause a strong overdetermination of CDT for serum samples with the transferrin D variants analyzed here. We suppose that such trisialo-tests always bear a higher risk for false positives regarding chronic alcohol abuse when transferrin D variants are present than tests using the CDT definition given by Stibler et al. *(16)*. The traces of trisialo-$Fe_2$-transferrin that occurred in the ChronAlcoI.D. microcolumn eluates constituted <5% of the total CDT. The intra- and interassay CVs of the whole ChronAlcoI.D. test were 9% and 11%, respectively *(17)*. Thus, the trisialo-$Fe_2$-transferrin traces do not significantly affect the CDT values, the CDT/transferrin ratio, or the corresponding upper reference limits indicating chronic alcohol abuse. The decision limits are affected, however, by the different analytic specificities and various recovery rates of the different CDT assays. Thus, CDT concentrations and CDT/transferrin ratios and upper reference limits from different CDT analysis methods must not be confused. Reliable decision criteria for serum CDT and the CDT/transferrin ratio determined by the ChronAlcoI.D. assay were reported recently *(17)*. In comparison with the transferrin pattern in the CDTect microcolumn effluxes (used for CDT determination) *(13)*, the CDT transferrin pattern in the ChronAlcoI.D. microcolumn eluate is more representative of that in the corresponding serum sample. Furthermore, we did not observe any overdetermination of CDT because of abnormal performance of the microcolumns in the ChronAlcoI.D. assay, as has been described for a few columns of the CDTect assay *(13)*. ChronAlcoI.D. microcolumns showed an apparently uniform elution behavior. The ChronAlcoI.D. test shows serum CDT as a percentage of total serum transferrin. The serum CDT and total transferrin concentrations are included. In our experience, this is an additional advantage of the ChronAlcoI.D. test in comparison with the other commercially available sets of reagents for CDT analysis. Whether absolute or relative CDT concentrations improve the diagnostic performance of CDT as a marker of chronic alcohol abuse has been discussed *(4, 24, 26–30)*. Indeed, increased total transferrin concentrations, e.g., because of pregnancy or iron-deficiency anemia, can potentially cause increased serum CDT concentrations and thus false positives with respect to alcohol abuse. Under these conditions, the CDT/transferrin ratio may be more specific for detection of chronic alcohol abuse than the CDT concentration. However, under the condition of reduced total transferrin concentrations, e.g., because of hemochromatosis, false positives are likely when the CDT/transferrin ratio is used instead of the CDT concentration. Recently, Helander *(31)* reported an overall similar diagnostic performance of absolute and relative CDT values. In our experience based on several thousand CDT analyses, both CDT and the CDT/transferrin ratio should be analyzed, and the total transferrin concentration should be taken into account *(17)*. In doing so, false positives attributable to abnormal total transferrin concentrations can be reduced as far as possible. It would be interesting to reevaluate the data published in the various clinical reports on CDT from this point of view.

In conclusion, with correct use, the initial CDT and non-CDT fractionation step involved in determination of CDT by the ChronAlcoI.D. assay is an efficient procedure for the elimination of non-CDT transferrins from the serum sample before CDT quantification in the final turbidimetric immunoassay. A standardization of CDT analysis is urgently needed.

We thank Sabine Motzny, Anne Warzecha, and Udo Weinheimer for excellent technical assistance, Bastian Hackler for graphic work, and Lloyd Allen Jones for stylistic emendations.

## References

1. Peter J, Unverzagt C, Engel W-D, Renauer D, Seidel C, Hösel W. Identification of carbohydrate deficient transferrin forms by MALDI-TOF mass spectrometry and lectin ELISA. Biochem Biophys Acta 1998;1380:93–101.
2. Landberg E, Påhlsson P, Lundblad A, Arnetorp A, Jeppsson J-O. Carbohydrate composition of serum transferrin isoforms from patients with high alcohol consumption. Biochem Biophys Res Commun 1995;210:267–74.
3. Henry H, Froehlich F, Perret R, Tissot J-D, Eilers-Messerli B, Lavanchy D, et al. Microheterogeneity of serum glycoproteins in patients with chronic alcohol abuse compared with carbohydrate-deficient glycoprotein syndrome type I. Clin Chem 1999;45: 1408–13.
4. Allen JP, Litten RZ, Anton RF, Cross GM. Carbohydrate-deficient transferrin as a measure of immoderate drinking: remaining issues. Alcohol Clin Exp Res 1994;18:799–812.
5. Arndt T. Carbohydrate-deficient transferrin (CDT): the most specific marker of chronic alcohol abuse available so far. J Lab Med 1999;23:392–406.
6. Stibler H, Kjellin KG. Isoelectric focusing and electrophoresis of the CSF proteins in tremor of different origins. J Neurol Sci 1976;30:269–85.

7. Bean P, Peter JB. Allelic D variants of transferrin in evaluation of alcohol abuse: differential diagnosis by isoelectric focusing-immunoblotting-laser densitometry. Clin Chem 1994;40:2078–83.
8. Hackler R, Arndt T, Kleine TO, Gressner AM. Effect of separation conditions on automated isoelectric focusing of carbohydrate-deficient transferrin and other human isotransferrins using the PhastSystem. Anal Biochem 1995;230:281–9.
9. Tagliaro F, Crivellente F, Manetto G, Puppi I, Deyl Z, Marigo M. Optimized determination of carbohydrate-deficient transferrin isoforms in serum by capillary zone electrophoresis. Electrophoresis 1998;19:3033–9.
10. Jeppsson J-O, Kristensson H, Fimiani C. Carbohydrate-deficient transferrin quantified by HPLC to determine heavy consumption of alcohol. Clin Chem 1993;39:2115–20.
11. Renner R, Kanitz R-D. Quantification of carbohydrate-deficient transferrin by ion-exchange chromatography with an enzymatically prepared calibrator. Clin Chem 1997;43:485–90.
12. Stibler H, Borg S, Joustra M. Micro anion exchange chromatography of carbohydrate-deficient transferrin in serum in relation to alcohol consumption (Swedish patent 8400587-5). Alcohol Clin Exp Res 1986;10:535–44.
13. Arndt T, Hackler R, Kleine TO, Gressner AM. Validation by isoelectric focusing of the anion-exchange isotransferrin fractionation step involved in determination of carbohydrate-deficient transferrin by the CDTect assay. Clin Chem 1998;44:27–34.
14. Foo Y, Rosalki SB. Carbohydrate deficient transferrin measurement. Ann Clin Biochem 1998;35:345–50.
15. Arndt T. Carbohydrate-deficient transferrin (CDT): different analytes under one name—standardization needed [Abstract]. Alcohol Alcohol 1999;34:486.
16. Stibler H. Carbohydrate-deficient transferrin in serum: a new marker of potentially harmful alcohol consumption reviewed. Clin Chem 1991;37:2029–37.
17. Arndt T, Behnken LJ, Martens B, Hackler R. Evaluation of the cut-off for serum carbohydrate-deficient transferrin as a marker of chronic alcohol abuse determined by the ChronAlcoI.D.™ assay. J Lab Med 1999;23:507–10.
18. Arndt T, Czylwik D, Hackler R, Helwig-Rolig A, Gilg T. Carbohydrate-deficient transferrin is not affected by serum separators. Alcohol Alcohol 1998;33:447–50.
19. Arndt T, Hackler R, Müller T, Kleine TO, Gressner AM. Increased serum concentration of carbohydrate-deficient transferrin in patients with combined pancreas and kidney transplantation. Clin Chem 1997;43:344–51.
20. Hackler R, Kleine TO. Modification of PhastSystem™ for the automated detection of oligoclonal bands in native cerebrospinal fluid by IEF with immunodetection. Lab Med 1991;15:185–92.
21. de Jong G, van Dijk JP, van Eijk HG. The biology of transferrin. Clin Chim Acta 1990;190:1–46.
22. van Noort WL, de Jong G, van Eijk HG. Purification of isotransferrins by concanavalin A Sepharose chromatography and preparative isoelectric focusing. Eur J Clin Chem Clin Biochem 1994;32:885–92.
23. de Jong G, van Eijk HG. Microheterogeneity of human serum transferrin: a biological phenomenon studied by isoelectric focusing in immobilized pH gradients. Electrophoresis 1988;9:589–98.
24. Renner F, Stratmann K, Kanitz RD, Wetterling T. Determination of carbohydrate-deficient transferrin and total transferrin by HPLC: diagnostic evaluation. Clin Lab 1997;43:955–64.
25. Lipkowski M, Dippelt L, Seyfarth M. Is there a benefit from including trisialo transferrin into the fraction of serum carbohydrate-deficient transferrin? [Abstract]. J Lab Med 1999;23:699.
26. Lesch OM, Walter H. New 'state' markers for the detection of alcoholism. Alcohol Alcohol 1996;31:59–62.
27. Huseby N-E, Nilssen O, Erfurth A, Wetterling T, Kanitz R-D. Carbohydrate-deficient transferrin and alcohol dependency: variation in response to alcohol intake among different groups of patients. Alcohol Clin Exp Res 1997;21:201–5.
28. Murawaki Y, Sugisaki H, Yuasa I, Kawasaki H. Serum carbohydrate-deficient transferrin in patients with nonalcoholic liver disease and with hepatocellular carcinoma. Clin Chim Acta 1997;259:97–108.
29. Rubio M, Caballería J, Deulofeu R, Caballería L, Gassó M, Parés A, et al. Carbohydrate-deficient transferrin as a marker of alcohol consumption in male patients with liver disease. Alcohol Clin Exp Res 1997;21:923–7.
30. Keating J, Cheung C, Peters TJ, Sherwood RA. Carbohydrate deficient transferrin in the assessment of alcohol misuse: absolute or relative measurements? A comparison of two methods with regard to total transferrin concentration. Clin Chim Acta 1998;272:159–69.
31. Helander A. Absolute or relative measurement of carbohydrate-deficient transferrin in serum? Experiences with three immunological assays. Clin Chem 1999;45:131–5.