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Validation by isoelectric focusing of the anion-exchange isotransferrin fractionation step involved in determination of carbohydrate-deficient transferrin by the CDTect assay

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Serum concentration of carbohydrate-deficient transferrin (CDT) is used for laboratory diagnosis of chronic alcohol abuse. Using isoelectric focusing for validation of the initial isotransferrin fractionation step involved in the determination of CDT by the CDTect assay, we found a complete in vitro iron saturation of transferrin and sufficient stability of the transferrin iron load during column passage; effective separation of non-CDT-isotransferrins and CDT-isotransferrins at the microcolumns; partial coelution of trisialo-Fe_2-transferrin, which did not significantly affect CDT measurement; partial retention of CDT-isotransferrins, especially disialo-Fe_2-transferrin, which may cause falsely negative results for CDT at the upper reference limits; good precision of the isotransferrin fractionation step; and no significant effects of low concentrations of serum protein and transferrin. We strongly urge standardization of CDT analysis and suggest isoelectric focusing for validation of CDT analysis methods and verification of odd results.

Isoelectric focusing detects various microheterogeneous forms of transferrin with differing isoelectric points. These various forms are the result of (*a*) altered protein moieties (genetic variants, e.g., transferrin-C, transferrin-D, and transferrin-B), (*b*) differences in iron load (no iron bound to transferrin, identified as Fe_0-transferrins; iron bound to the C-terminal or N-terminal binding site, Fe_{1C}- or Fe_{1N}-transferrins; both binding sites loaded with iron, Fe_2-transferrins), and (*c*) different carbohydrate chains, with 0 to 8 sialic acid residues (asialo-, monosialo-,. . . , octasialo-transferrins) *[1–3]*. Increased concentrations of sialic acid-deficient transferrins (a-, mono-, and mainly disialo-transferrin) are detected in serum of alcoholics *[4]*. The serum concentration of these isotransferrins, collectively referred to as carbohydrate-deficient transferrin (CDT) *[4]*, is used for detection and follow-up of chronic alcohol abuse *[5–15]*.

Specific chemical reactions or antibodies for analysis of CDT are not available so far. CDT is usually determined, therefore, after separation from more-highly sialylated non-CDT-isotransferrins (tri-, tetra-, . . . , octasialo-transferrin) by chromatographic *[16–21]* or electrophoretic *[5, 22–25]* methods. To reduce the number of microheterogeneous transferrin forms and to preclude coelution or cofocusing of transferrins with different contents of sialic acid and iron but the same isoelectric point (e.g., disialo-Fe_2-transferrin as the main CDT-isotransferrin and tetrasialo-Fe_1-transferrin as the main non-CDT-isotransferrin), analysis is usually done after in vitro iron saturation of the transferrin. With this sample pretreatment, only Fe_2-transferrins (and no Fe_1- and Fe_0-transferrins) are present in the serum sample.

Commercially available sets of reagents for determination of CDT use in vitro iron saturation of transferrin to eliminate Fe_1- and Fe_0-transferrins and anion-exchange microcolumns to fractionate (separate) the non-CDT- and the CDT-isotransferrins. Subsequent quantification of the CDT-isotransferrins in column effluxes or eluates is done by RIA [CDTect-RIA (Pharmacia & Upjohn), and %CDT (AXIS, Oslo, Norway)], enzyme immunoassay (CDTect-EIA; Pharmacia & Upjohn), or turbidimetrically (CDTri-TIA; AXIS). Because anti-transferrin (and not anti-CDT) antibodies are used in these assays, the presence of non-CDT-isotransferrins in the column effluxes can lead to distinct overestimation of CDT. No quality-control

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material or external quality-control program for analysis of CDT is available so far, despite the increasing use of CDT, e.g., in forensic and employment medicine *[26–28]*.

A unique definition of CDT is still missing. Until recently, all commercially available methods for determination of CDT and nearly all of the studies published summarized asialo-, monosialo-, and disialo-Fe_2-transferrin as CDT *[4, 5–19, 21–27]*. Now, a set of reagents incorporating trisialotransferrin in determination of CDT (CDTri-TIA) has been commercially launched. The same company offers a second set of reagents that does not include trisialotransferrin (%CDT). The directions for use of CDTect and %CDT give no clear information as to whether trisialotransferrin is completely, in part, or not at all determined with the asialo-, monosialo-, and disialo-Fe_2-transferrin. Consequently, comparison and interpretation of CDT values are more and more difficult for the clinical chemist, the clinician, and the jurist.

The aim of our study was to investigate a widely distributed commercially available set of reagents, CDTect-RIA, for reliability of the initial anion-exchange isotransferrin fractionation step involved in the determination of CDT. We analyzed the isotransferrin patterns of serum samples by isoelectric focusing after each intermediate processing step. We wanted to get information about (*a*) the completeness of the in vitro transferrin iron-saturation step of the CDTect assay, which is used to eliminate the Fe_1- and Fe_0-transferrins; (*b*) the stability of the transferrin iron load during the passage of the serum sample through the chromatographic column, so as to prevent coelution of non-CDT- and CDT-isotransferrins of different iron contents; (*c*) the efficiency of the initial isotransferrin fractionation step at the anion-exchange microcolumns, and the fate of trisialotransferrin; (*d*) the reproducibility of the initial isotransferrin fractionation step, and (*e*) possible effects of variations of serum protein and transferrin concentrations on the CDTect assay performance.

Materials and Methods

MATERIALS

All chemicals were of analytical grade and obtained from Merck except: Pharmalytes 5–6 (Pharmacia/LKB) and polyclonal antibodies (purified immunoglobulins) to transferrin (Dako).

SERUM SAMPLES

All procedures were in accordance with the Helsinki Declaration of 1975, as revised in 1983. Only surplus serum sample volumes from routine employment and forensic investigations and from patients undergoing alcohol withdrawal treatment were used. For all subjects, an 18-variable clinical laboratory profile was established. The patients had no signs of bacterial infection or inflammation, and no infusions had been given. Blood was drawn after an overnight fast into sterile gel-tubes (Sarstedt) in which the gel barrier consisted of an polymerized acrylic resin. After clotting at room temperature for 30 min, the blood samples were centrifuged at 2000*g* for 10 min at 4 °C. To avoid contamination of the serum samples with microorganisms, the serum was removed with disposable pipettes. The serum was split into one aliquot for routine laboratory analysis and another aliquot (surplus serum volume) for determination of CDT. The surplus serum volumes were immediately transferred into sterile, leak-proof plastic containers (1.2-mL Nalgene Cryotubes System) and stored at −70 °C. By this means, serum samples were frozen within 30 min after centrifugation. They were thawed only once, on the day of analysis.

METHODS

Assay of serum CDT concentration. The serum concentration of CDT was determined by CDTect-RIA in accordance with the manufacturer's instructions. In short, after in vitro iron saturation of transferrin (mixing 50 μL of serum sample, 200 μL of ferric citrate solution, and 1 mL of buffer) and adsorption of isotransferrins with isoelectric point values <5.7 (non-CDT-isotransferrins) on the anion-exchange microcolumns, the isotransferrins with isoelectric point >5.7 (CDT-isotransferrins) in the column effluxes were determined by means of a competitive, double-antibody immunoadsorbent assay.

Quality control for the whole CDTect assay was done in accordance with the Guidelines of the Federal German Medical Association. In each run, serum pool aliquots with CDT values near the upper reference limits (20 U/L for men and 30 U/L for women) and a control sample supplied with the set of reagents were used for internal quality control, being placed at the beginning and end of each run. Control and serum samples were analyzed in duplicate. Intra- and interassay variations for the entire CDTect assay were 10% and 17%, respectively. Quality-control data were documented on quality-control sheets. No external quality-control program was available.

For validation by isoelectric focusing of the initial isotransferrin fractionation step used in determination of CDT by CDTect-RIA, we retained 10-μL aliquots of the original column effluxes. Column eluates were obtained by eluting the columns with 0.5 mL of 2 mol/L NaCl solution.

Isoelectric focusing. Qualitative isoelectric focusing on PhastSystem™ (Pharmacia/LKB) was done as described previously *[22]*, as modified *[29]*. In short, polyacrylamide gels, pH 5–6 (total acrylamide content T = 5%, cross-linker content C = 3%; 43 × 50 × 0.45 mm; Pharmalyte 5–6® diluted 1:16), adhering to a plastic support film (GelBond™ PAG film; Biozym-Diagnostik, Hameln, Germany), were prepared by us. After prefocusing of the gels (75 V · h), 1 μL of pretreated sample was applied with use of Sample Applicator™ 8/1 (Pharmacia/LKB) *[22, 29]*. Separation was performed for 200 V · h.

Sample pretreatment for isoelectric focusing. To achieve optimal specificity and sensitivity for detection of CDT-

isotransferrins, we diluted the iron-saturated serum samples and NaCl eluates to a transferrin concentration of 7.5 mg/L (final dilution factor 200- to 500-fold, depending on the original transferrin concentration of the sample). The column effluxes were analyzed in the original state (dilution factor 25-fold, after the initial serum transferrin iron-saturation step). By this means, distinctly different intensities (Fig. 1, left panel) and peak heights (Fig. 1, right panel) for CDT-isotransferrin bands were obtained for serum samples and eluates on the one hand and for effluxes on the other.

Immunofixation. Immunofixation was carried out as described elsewhere *[22, 29]*. In short, gels were covered with polyclonal antibodies against transferrin, covered with a plastic foil equal to the size of the gel to achieve an even distribution of the antibody solution, and incubated at room temperature in a moist chamber. Specificity of the transferrin antibodies was tested as described *[22]*. Unprecipitated proteins were removed by washing with 150 mmol/L sodium chloride solution overnight.

Visualization and evaluation of the transferrin bands. The isotransferrin bands were visualized by silver staining in the PhastSystem Development Unit™ as described previously *[22, 29]*. Transferrin bands were identified by comparison with identically treated isotransferrin preparations or by parallel analysis of cerebrospinal fluid samples, showing asialo- to hexasialo-Fe_2-transferrin bands (lane CSF in Figs. 1–3). Gels were air-dried and stored for documentation.

Densitometric evaluation of the isotransferrin bands (Figs. 1, right, and 2) was done with an Elscript 400 densitometer (Hirschmann Gerätebau, Unterhaching, Germany) at 523 nm, with a diaphragm aperture of 0.13 mm.

Other assays. Serum protein concentration was determined by use of the biuret method with a Hitachi 747 analyzer and reagents from Boehringer Mannheim. Serum transferrin concentration was measured immunonephelometrically with a Behring Nephelometer Analyzer (BNA) and reagents from Behring. Internal and external quality con-

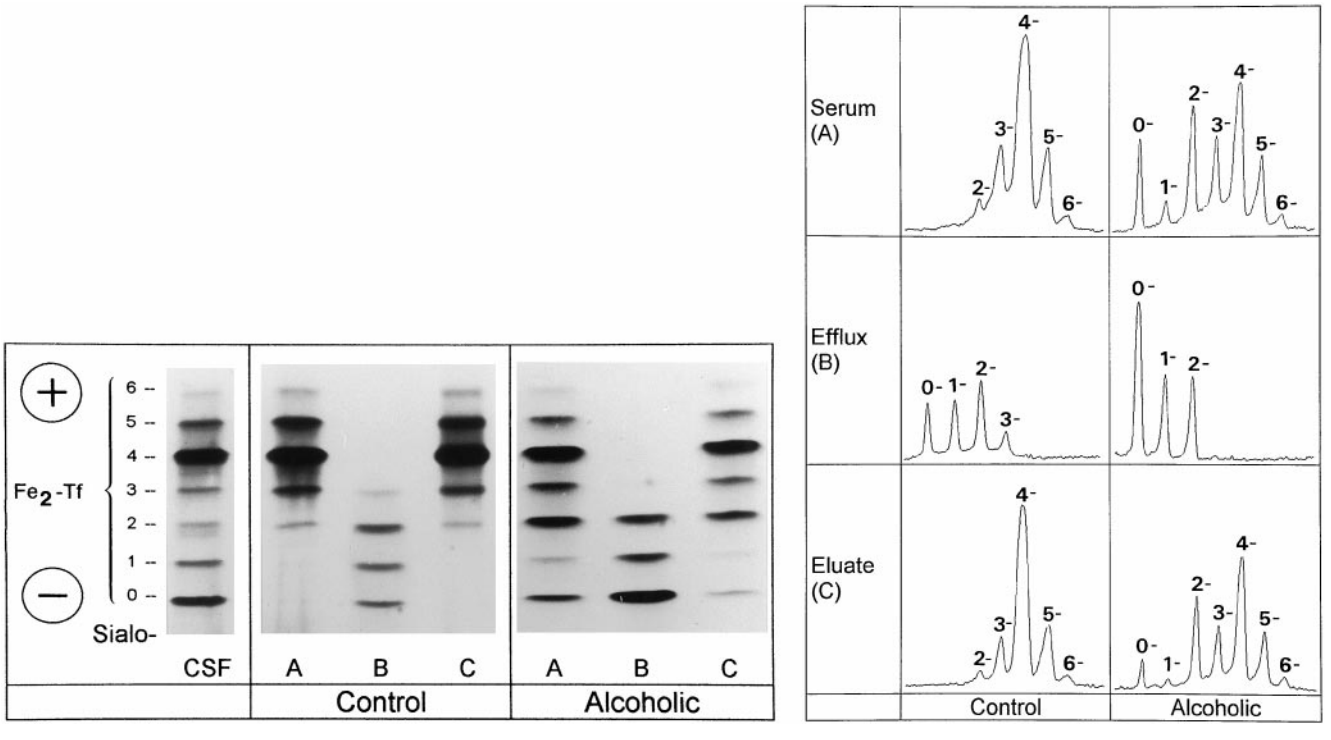

Fig. 1. Testing the efficiency of the in vitro transferrin iron saturation, the transferrin iron-load stability, and the separation of non-CDT- and CDT-isotransferrins by CDTect: isoelectric focusing isotransferrin band patterns (*left panel*) and the corresponding densitograms (*right panel*).

Lanes *A*: Isotransferrin band patterns for serum from a healthy female control subject (CDT = 24 U/L) and an alcoholic (CDT = 153 U/L) after the in vitro transferrin iron saturation step. *Lanes B*: Isotransferrin patterns in the corresponding anion-exchange microcolumn effluxes. *Lanes C*: Isotransferrin patterns in the corresponding anion-exchange microcolumn NaCl eluates. *Lane CSF*: Isotransferrin band pattern of an in vitro iron-saturated cerebrospinal fluid used for identification of the transferrin bands in *lanes A-C*. The presence of only Fe_2- (and no Fe_1- and Fe_0-) transferrins in *lanes A-C*, *left panel*, proves the efficiency of the in vitro transferrin iron-saturation step and the sufficiently stable transferrin iron load during anion-exchange microcolumn passage. Higher-sialylated, non-CDT-isotransferrins (mainly tetrasialo-Fe_2-transferrin) were not found in the effluxes (*lanes B*), thereby pointing to a reliable retention of these transferrins on the anion-exchange column. Traces of trisialo-Fe_2-transferrin did not significantly affect the final CDT result. The presence of disialo-Fe_2-transferrin and, for the alcoholic, asialo-Fe_2-transferrin in the eluates (*lanes C*) reflects partial retention of these at the anion-exchange column, causing incomplete CDT recovery. This retention was different for each CDT-isotransferrin. [*Note*: See text regarding overload of the tetrasialo-Fe_2-transferrin fraction. The intensity (*left panel*), peak height, or peak area (*right panel*) of the tetrasialo-Fe_2-transferrin bands therefore does not correlate with the transferrin content of this fraction. Because of variations in the final dilution factors (200–500-fold for serum and NaCl eluates and 25-fold for effluxes), only the isotransferrin peak-height ratios but not the peak heights of *lanes A* and *C* in the *right panel* are comparable with those of *lanes B*.]

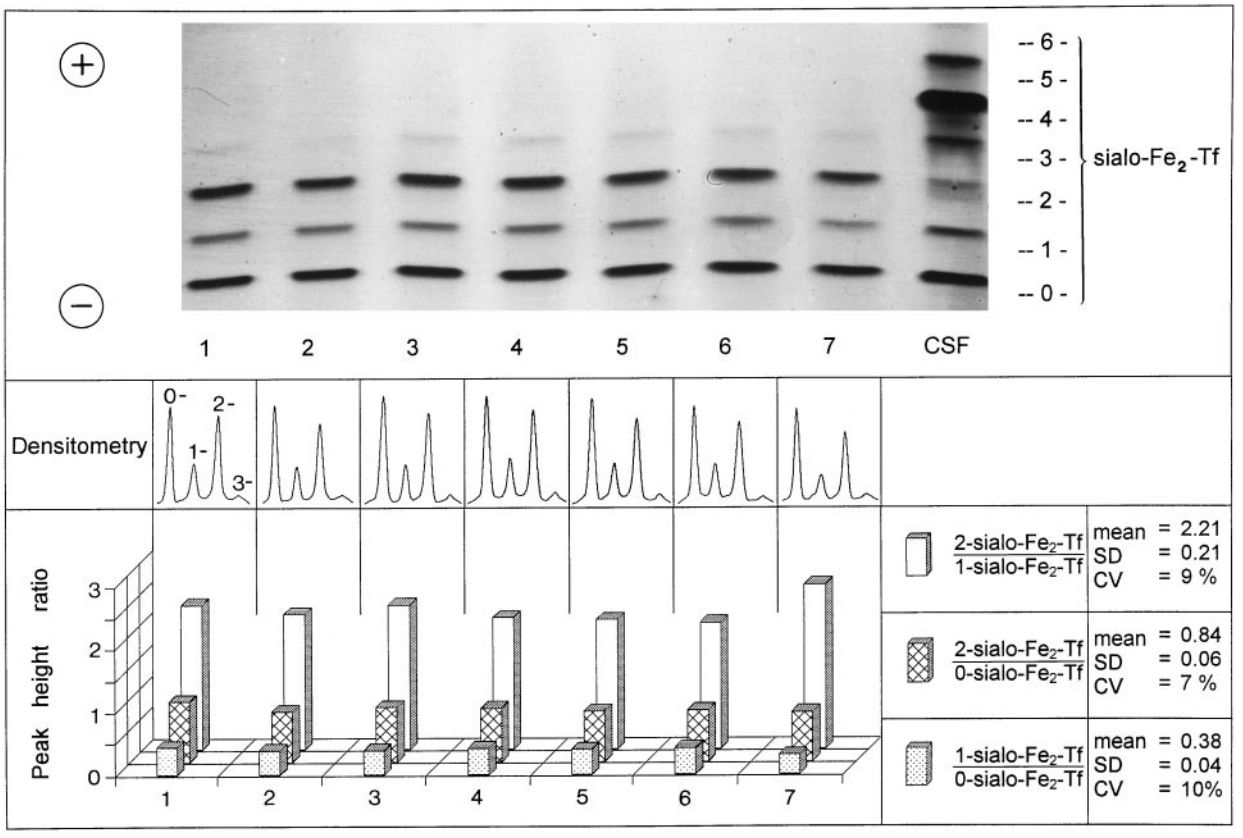

Fig. 2. Testing the intraassay imprecision of the CDTect initial isotransferrin fractionation step.

Lanes 1–7 show characteristic isoelectric focusing band patterns and the corresponding densitograms of seven column effluxes, which were obtained by sevenfold identical processing of the serum samples in accordance with the CDTect procedure. In calculating the mean, SD, and CV of the peak-height ratios of *lanes 1–7*, we found CVs of ≤10%, which are very close to that of the whole CDTect-RIA assay (10%) and point to a uniform column performance.

trol was done in accordance with the Guidelines of the Federal German Medical Association.

Results

ISOELECTRIC FOCUSING

Detailed information concerning the isoelectric focusing procedure used in this study for evaluation of the initial isotransferrin fractionation step of the CDTect assay is given in previous reports *[22, 29]*. In interpreting the isotransferrin band patterns in Figs. 1–3, one must consider the following: In adjusting the sensitivity of the isoelectric focusing for detection of CDT-isotransferrins, we accepted an overload of the ~100-fold greater concentration of tetrasialo-Fe_2-transferrin fraction. The intensity of the tetrasialo-Fe_2-transferrin band thus does not correlate with the amount of this isotransferrin, making quantification of CDT or measurement of the CDT/transferrin ratio in the isoelectric focusing gel impossible.

CDTECT ASSAY

In vitro transferrin iron saturation. In the case of incomplete in vitro iron saturation of transferrin, bands of Fe_0- and Fe_1-transferrins should be detected by isoelectric focusing but will disappear after addition of further amounts of Fe^{3+}. We did not find bands of Fe_0- and Fe_1-transferrins in the iron-saturated serum samples. Furthermore, we compared the isoelectric focusing isotransferrin band patterns of serum samples after the Fe^{3+} treatment according to the CDTect test instructions and after Fe^{3+} treatment *[22]*. Varying the Fe^{3+} treatment did not affect the number and position of the transferrin bands or the isotransferrin peak-height ratios. Both Fe^{3+}-treatment procedures yielded isoelectric focusing isotransferrin band patterns similar to those in lanes A of Fig. 1, i.e., di-, tri-, tetra- (main fraction), penta-, and hexasialo-Fe_2-transferrins for healthy controls with normal alcohol intake (<40 g of ethanol per day). For alcoholics, a stronger disialo-Fe_2-transferrin band and additional bands of monosialo- and asialo-Fe_2-transferrin were found, reflecting the effects of increased alcohol consumption.

Transferrin iron-load stability during column passage. If transferrin iron is lost during column passage, bands of Fe_0- and Fe_1-transferrins should be detected in the column effluxes. Moreover, these bands should disappear after addition of further amounts of Fe^{3+} to the effluxes. Of 130 column effluxes, 117 showed isoelectric focusing trans-

ferrin band patterns in which the only bands present were Fe_2-transferrins; no bands for Fe_0- or Fe_1- transferrins and no smear were present (lanes B of Fig. 1). A smear can result by the overlapping of several transferrins with different iron and sialic acid contents because of transferrin iron loss *[22]* during passage through the anion-exchange column.

For 13 column effluxes, we obtained isotransferrin band patterns similar to that of lane C in Fig. 3, with CDT-isotransferrin bands (asialo-, monosialo-, and disialo-Fe_2-transferrin) and additional more-cathodic bands (indicated by the arrowheads). The cathodic bands disappeared after addition of further amounts of Fe^{3+} to these column effluxes. At the same time, new bands of more-highly sialylated isotransferrins, possibly cofocused with CDT-isotransferrins because of incomplete transferrin iron saturation, did not appear. The resulting isoelectric focusing patterns for isotransferrin bands were similar to those of lanes B of Fig. 1 (left). These findings indicate a weak iron loss from CDT-isotransferrins during column passage in 13 of 130 columns. In none of the effluxes did we find higher-sialylated Fe_0- or Fe_1- transferrins (tetra-, penta-, hexa-, hepta-, and octasialotransferrins). From this we conclude that the transferrin iron saturation was complete and sufficiently stable, allowing a reliable fractionation of non-CDT- and CDT-isotransferrins at the anion-exchange microcolumns. Thus, with correct use of the CDTect assay, we conclude that transferrin iron-load stability is sufficient during the isotransferrin fractionation step.

Annotation. Effluxes of expired columns, or of columns that had been in contact with air for 1 week (inappropriately stored surplus columns from the previous analysis runs), contained distinct amounts of Fe_0- or Fe_1-transferrins and higher-sialylated isotransferrins (e.g., tetrasialo-Fe_2-transferrin), which caused CDT overestimations in the final RIA procedure. The corresponding band patterns (not shown) were similar to that in lane B of Fig. 3.

Efficiency of the isotransferrin fractionation at the anion-exchange microcolumns. The efficiency of the adsorption of higher-sialylated, non-CDT-isotransferrins at the anion-exchange column, as well as the elution behavior of the CDT-isotransferrins, was investigated by isoelectric focusing analysis of the original microcolumn effluxes and of additional 2 mol/L NaCl eluates. Higher-sialylated, non-CDT-isotransferrins, especially tetrasialo-Fe_2-transferrin as the main isotransferrin, were found in only 2 of 130 column effluxes (lane B of Fig. 3). However, these 2 columns were already showing a distinctly decelerated elution speed during column equilibration, attributable to clogged filters at the bottom of the columns. Of 130 column effluxes, 117 contained traces [lanes B of Fig. 1(left), lanes 1–7 of Fig. 2), 1 contained distinct amounts (lane A of Fig. 3), and 12 no amounts of trisialo-Fe_2-transferrin. These trisialotransferrin amounts, however, were quantitatively less important (<10% of the final CDT result) and were within the analytical imprecision of 10% for the whole CDTect assay.

The recovery of the CDT-isotransferrins was qualitatively assessed by eluting the CDTect microcolumns with 2 mol/L NaCl solution and isoelectric focusing analysis of the eluates. Typical isoelectric focusing isotransferrin band patterns of these eluates are shown in lanes C of Fig. 1 (left). In all eluates we found higher-sialylated, non-CDT-isotransferrins (tri-, tetra-, penta-, and hexasialo-Fe_2-transferrin), which were correctly adsorbed on the anion-exchange column. In all eluates, however, we also found disialo-Fe_2-transferrin (the main CDT-isotransferrin) and, for alcoholics, asialo-Fe_2-transferrin (lanes C of Fig. 1, left). This indicates a partial retention of these isotransferrins at the anion-exchange column and thus incomplete recovery of CDT. Because of the different final dilution factors (see *Materials and Methods*), the isotransferrin peak heights (Fig. 1, right) are comparable only between serum (lanes A) and eluate (lanes C), but not between serum and efflux (lanes B) or between efflux and eluate (lanes B and C). For the alcoholic (CDT = 153 U/L; Fig. 1, right), peak-height ratios of disialo-Fe_2-transferrin/asialo-Fe_2-transferrin >1 were obtained for serum and eluate (lanes A and C) and

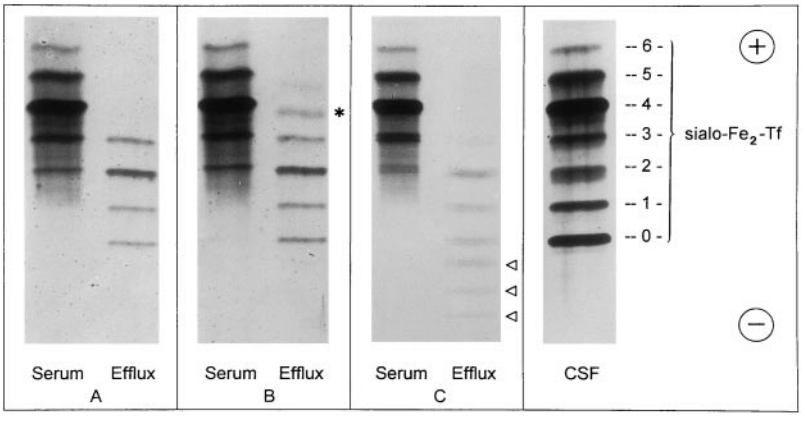

Fig. 3. Rare cases of insufficient separation of CDT- and non-CDT-isotransferrins at the anion-exchange microcolumns.

Of 130 column effluxes, one showed exceptionally high amounts of trisialo-Fe_2-transferrin (*A*) and two showed additional fractions of non-CDT-isotransferrins (tri- and tetra-, indicated by *, and higher-sialylated isotransferrins; *B*). The corresponding columns showed a distinctly delayed elution velocity, with increased CVs in duplicate measurements, and were eliminated from routine analysis. Bands of iron-deficient transferrins (indicated by *arrowheads* in *C*), resulting from partial iron loss from CDT-isotransferrins during or after column passage and not from coelution of higher-sialylated non-CDT-isotransferrins, were observed in 13 of 130 column effluxes but had no effect on the final CDT value.

<1 for the efflux (lane B). This switch is because of a lesser retention of asialo-Fe_2-transferrin than of disialo-Fe_2-transferrin at the anion-exchange column.

Reproducibility of the initial isotransferrin fractionation step. The within-run reproducibility of the CDTect initial isotransferrin fractionation step was tested by processing 7 times serum samples with normal and increased CDT. Calculating the mean, SD, and CV 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 an imprecision of ≤10% (Fig. 2), which was very close to that of the whole CDTect assay (10% intraassay).

Effects of various serum protein and transferrin concentrations on microcolumn function. Various serum protein and transferrin concentrations were achieved by dilution of serum samples of healthy controls and alcoholics with 150 mmol/L NaCl solution. The resulting protein and transferrin concentrations were 32–81 g/L (mean 55 g/L; n = 14) and 1.1–3.9 g/L (mean 2.3 g/L; n = 14), respectively. The recovery of CDT in the diluted serum samples ranged from 87% to 116% (mean 100%, n = 7). The correlation equation between calculated (x) and measured CDT (y) was $y = 1.0x - 0.2$ ($r = 0.994$). This indicates a sufficient stability of the microcolumn anion-exchange reactions to low serum protein and transferrin concentrations, and a sufficient linearity of CDT measurement over the clinically significant serum concentration ranges of protein, transferrin, and CDT.

Discussion

The term carbohydrate-deficient transferrin, or CDT, refers to all transferrin components that lack two or four of their terminal trisaccharides and whose charge corresponds to disialo- (and to a lesser degree, mono- and asialo-) transferrin *[4]*. Serum concentration of CDT is increasingly used in employment and forensic medicine and as a decision criterion for reissuing drivers' licenses *[26–28]*.

The main problem of CDT analysis arises from the fact that the isotransferrins show very similar structural and chemical properties and extremely different serum concentrations *[1–3]*. Thus, the CDT-isotransferrins (usually <30 mg/L; asialo-, mono-, and disialo-Fe_2-transferrin) occur within a matrix containing 2200–4400 mg/L of higher-sialylated, non-CDT-isotransferrins, especially tetrasialotransferrin. Reliable chromatographic or electrophoretic separation of non-CDT- and CDT-isotransferrins is complicated by identical or very similar isoelectric point values of isotransferrins that have different iron and sialic acid contents (minimal isoelectric point difference 0.1 pH units) and by the presence of genetic variants *[4, 5]*. Analysis is therefore usually done after elimination of Fe_0- and Fe_1-transferrins by in vitro iron saturation of transferrin. As we showed previously by isoelectric focusing *[22]*, transferrin iron loss can occur under nonoptimal focusing conditions, such that non-CDT- and CDT-isotransferrins are cofocused. This transferrin iron loss may also occur under nonoptimal conditions at anion-exchange microcolumns, which are widely used in (commercially available) tests for determination of CDT.

The isotransferrin patterns in the anion-exchange microcolumn effluxes of the commercially available CDTect assay are studied here the first time. The data obtained in this study provide evidence for (*a*) complete elimination of Fe_0- and Fe_1-transferrins in the in vitro transferrin iron saturation step, (*b*) sufficient transferrin iron-load stability during column passage, and (*c*) reliable retention of non-CDT-isotransferrins (especially tetrasialo-Fe_2-transferrin). Most of the column effluxes contained, besides asialo-, monosialo-, and disialo- Fe_2-transferrin, traces of trisialo-Fe_2-transferrin, which by definition does not belong to CDT *[4]*. Complete separation of trisialotransferrin from CDT by anion-exchange chromatography is, however, very difficult, owing to the very close isoelectric points of disialo-Fe_2-transferrin and tetrasialo-Fe_2-transferrin. In the original method of Stibler et al. (reviewed in *[4]*), all isotransferrins with isoelectric points >5.65 were analyzed. However, the stability of this microcolumn isotransferrin separation was insufficient, owing to the very low ionic strength of the elution buffer *[4]*. In their modified method, which is the basis for CDTect, "mainly components with isoelectric point values >5.7, and only a small amount of the isotransferrin with pI 5.7" are measured *[4]*. Our results, however, demonstrate that traces of trisialo-Fe_2-transferrin (<10% of CDT) are also present in the column effluxes. These contaminations were within the analytical imprecision of the whole assay and did not significantly affect the final CDT result.

Recently, Heggli et al. *[20]* reported increased trisialo-Fe_2-transferrin concentrations in serum samples of alcoholics. Including this isotransferrin in determination of CDT improved the "distinction between social drinkers and heavy drinkers" and the authors conclude: "Further investigations to establish the best clinically analytical composition of transferrin variants in clinical testing of long-term alcohol intake may be fruitful" *[20]*. Until now, a final decision as to whether trisialotransferrin should be incorporated into CDT is not possible. Definitely, the manufacturer of sets of reagents for determination of CDT must inform the users which isotransferrins are being analyzed as CDT. This information is missing in, e.g., the package inserts of the CDTect and the %CDT assays.

Whether trisialotransferrin is analyzed together with asialo-, monosialo-, and disialotransferrin is not only of scientific interest but also has practical implications. Under ordinary conditions, trisialotransferrin represents ~9% of the total serum transferrin concentration, in comparison with the 3% of disialo-, monosialo-, and asialotransferrin combined *[30]*. Thus, complete, partial, or no coelution of this isotransferrin strongly affects such

items as the upper reference limits that indicate chronic alcohol abuse *[31]*. It may also affect the diagnostic sensitivity and specificity.

Of 130 columns, only 1 showed extraordinary amounts of trisialo-Fe_2-transferrin eluted (lane A of Fig. 3) and only 2 showed tetrasialo-Fe_2-transferrin as the main non-CDT-isotransferrin (lane B of Fig. 3). However, during column equilibration, these columns already showed a reduced elution speed. For such columns, increased CVs for duplicate measurements were obtained. We suggest a visual check of the anion-exchange microcolumn elution behavior during the initial isotransferrin fractionation step when determining CDT by the CDTect method. Columns with abnormal elution behavior bear the risk of CDT overestimation and must be sorted out. Although the column quality was very uniform, as indicated by CV $<10\%$ for the CDT-isotransferrin peak-height ratios in the column effluxes (Fig. 2), we suggest a duplicate analysis for each serum sample. This would prevent incorrect determination of CDT and false results owing to nonoptimal performance of a single column. Variations of $>10\%$ for the duplicate CDT measurement should be validated by repetition of the analysis or by isoelectric focusing as the comparison method. In our experience and with correct use of the CDTect assay, the risk of overestimation of CDT on account of analytical nonspecificities, e.g., partial or complete coelution of tetrasialo-Fe_2-transferrin with the CDT-isotransferrins, is low.

A point that needs further investigation is the incomplete and variable recovery of the different CDT-isotransferrins in the column effluxes. Given the total lack of negatively charged sialic acid residues for asialo-Fe_2-transferrin, we conclude this isotransferrin showed the lowest affinity to the anion-exchange microcolumn and the best recovery. Owing to 2 negatively charged sialic acid residues, disialo-Fe_2-transferrin had a stronger affinity to the anion-exchange material, resulting in partial retention at the microcolumn (lanes C of Fig. 1) and reduced recovery in the microcolumn effluxes. This may be critical, because this isotransferrin represents the main fraction of CDT. For CDT results at the gender-specific upper reference limits, this may cause falsely negative results. Obviously, in adjusting the assay for the greatest specificity (minimizing the risk of coelution of tetrasialo-Fe_2-transferrin with CDT-isotransferrins, which would cause overestimations and falsely positive results), an incomplete recovery of CDT (and in some cases falsely negative results for real CDT concentrations at the gender-specific upper reference limits) was accepted. Thus, modifying the original method of Stibler et al. as described above resulted in lower upper reference limits for the CDT values that indicate greater alcohol consumption *[4]*. Accordingly, it is important not to confuse CDT values and upper reference limits for CDT that were obtained by different analysis methods.

An incomplete CDT recovery could be accepted if the recovery is constant over the clinically important concentration range. This was tested by twofold (and in some cases sixfold; data not shown) dilution of serum samples from controls and alcoholics. The correlation coefficient of 0.994 between calculated and measured CDT points to a sufficient linearity of the CDT recovery in the clinically important serum concentration ranges of protein, transferrin, and CDT.

A serious drawback of the isotransferrin fractionation by anion-exchange reactions is its failure to detect genetic transferrin variants that can cause false-positive (transferrin-D) or false-negative (transferrin-B) results. Further studies are required for investigating the possible effects of genetic transferrin variants or transferrin phenotypes on CDTect performance. For a satisfactory evaluation of this problem, analysis of serum samples to which have been added various amounts of appropriate isotransferrin preparations (which are thus far hard to obtain) is necessary. This was beyond the scope of this study. Possibly, individualized reference values, as recently suggested by Borg et al. for long-term monitoring of alcohol-dependent patients *[32]*, may be useful for improving the diagnostic specificity and sensitivity of CDT in the case of genetic transferrin variants.

In conclusion, with correct use, the initial isotransferrin fractionation step involved in determination of CDT by the CDTect method was efficient for elimination of non-CDT-isotransferrins. According to personal information from the manufacturer, the same microcolumns are used in the CDTect-RIA and CDT-EIA reagent sets. The results obtained in this study for the determination of CDT by the CDTect-RIA method should therefore also be valid for CDTect-EIA. We recommend a procedure similar to that described here for analytical evaluation of other appropriate CDT analytical methods. Isoelectric focusing should be used for detection of transferrin variants or transferrin phenotypes and for validation of unusual or unexpected results obtained by other procedures. The data presented here indicate an urgent need for standardization of CDT analysis.

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