Semiautomated procedures for evaluation of carbohydrate-deficient transferrin in the diagnosis of alcohol abuse

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Carbohydrate-deficient transferrin (CDT) may now be the most valuable biological marker for diagnosis of alcohol abuse. We compared the diagnostic performance of two new CDT tests, Axis %CDT turbidimetric immunoassay (TIA) and Axis %CDT HPLC, against Specialty Laboratories' isoelectric focusing/immunoblotting/laser densitometry (IEF/IB/LD). Both Axis tests include one-half the concentration of trisialotransferrin isoforms in their CDT quantitation schemes. Considering an alcohol abuse prevalence of 7%, Axis %CDT TIA shows a sensitivity of 87% at 98% specificity and a positive predictive value (PPV) of 0.75; %CDT HPLC shows a sensitivity of 87% at 100% specificity for a PPV of 1, and the IEF/IB/LD shows 81% sensitivity at 94% specificity for a PPV of 0.5. All three CDT tests show the same negative predictive value (0.98). Both Axis procedures perform better than IEF/IB/LD in the diagnosis of alcohol abuse; %CDT TIA is available in several semiautomated, cost-effective formats.

INDEXING TERMS: carbohydrate-deficient transferrin • alcohol abuse • diagnosis

Carbohydrate-deficient transferrin (CDT) 3 is currently the most valuable biological marker available for diagnosis of sustained alcohol abuse [1, 2]. In well-defined populations of alcohol abusers (>60 g of alcohol daily for 7–10 consecutive days) and sex, race, and age-matched control nondrinkers, sensitivity and specificity of CDT are >80% and >90%, respectively [1–5]. However, CDT tests that perform very well in a highly selected sample perform less acceptably in more heterogeneous groups such as those encountered in primary care or the general population. In unselected populations like that in the Svalbard study of young Finnish University students and patients admitted consecutively to a medical department at Oslo, sensitivity of CDT was 22–69%; specificity was almost always >90% [2, 6–8]. The current technology supports the use of CDT as a diagnostic tool only in populations with a reasonably high prevalence of alcohol abusers [3–5].

The only CDT procedure commercially available in the US is the Specialty Laboratories (Santa Monica, CA) isoelectric focusing/immunoblotting/laser densitometry (IEF/IB/LD) method, introduced during the early 1990s [9]. This and Pharmacia’s CDTect, used widely in Europe but available in the US for research purposes only, are pioneer CDT tests used extensively in several clinical settings [1, 3–8]. Both procedures use a charge-based method to separate CDT molecules containing two, one, or no sialic acids, followed by detection in different immunoassays; IEF/IB/LD measures CDT as a ratio of total transferrin, whereas CDTect measures absolute values of CDT.

We describe two new laboratory procedures for evaluation of CDT, Axis (Oslo, Norway) %CDT turbidimetric immunoassay (TIA) and Axis %CDT-HPLC, and compare their performance to that of the pioneer IEF/IB/LD. In addition to measuring (all or a fraction of) the disialo-, monosialo-, and asialotransferrins, both these new procedures include half the concentration of trisialotransferrin in their CDT quantitation schemes and report CDT results as a relative amount to total serum transferrin. The relevance of these new evaluation methods is discussed in the context of their implications for the widespread use of CDT because inclusion of the trisialotransferrin fraction

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3 Nonstandard abbreviations: CDT, carbohydrate-deficient transferrin; %CDT TIA, %CDT turbidimetric immunoassay; IEF/IB/LD, isoelectric focusing/immunoblotting/laser densitometry; DU, densitometry units; PPV, positive predictive value; and NPV, negative predictive value.

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increases the accuracy in the diagnosis of sustained alcohol usage. The Axis %CDT TIA is available in a manual format as well as several semiautomated, cost-effective formats.

**Materials and Methods**

### PATIENTS AND CONTROLS

Seventy-eight serum specimens (58 obtained from Axis and 20 from Specialty Laboratories) were analyzed for CDT. All specimens from Specialty were obtained from African Americans, an ethnic group with a high incidence of genetic D transferrin variants and a reported source of false positives for CDT [10]. The gender, race, and age as well as alcohol consumption of patients and control groups were: group 1, 7 abstinent Caucasian pregnant women, average age 32 years; group 2, total abstainers for many years (n = 8), 3 men and 5 women, all Caucasians with no liver disease, average age 68 years; group 3, social drinkers (n = 33), 17 men, including 5 African Americans, and 16 women, including 8 African Americans, average age 38 years (social drinkers consumed <40 g of alcohol daily for several years, had no known liver disease, and included two genetic transferrin variant CD1 carriers); group 4, alcohol abusers, 23 men, including 4 African Americans, and 9 women, including 3 African Americans, average age 49 years (alcohol consumption in this group, represented by patients with and without liver disease, was 100–400 g/day).

After CDT quantitation by %CDT TIA and %CDT HPLC, all 58 specimens obtained from Axis were shipped frozen as blind samples to Specialty for analysis by IEF/IB/LD. After CDT quantitation by Specialty’s IEF/IB/LD, all 20 specimens derived from African Americans were shipped frozen as blind samples to Axis for analysis by %CDT TIA and %CDT HPLC. Serum aliquots were stored for <3 months at −20 °C before analysis; only 76 of the 78 specimens were available for analysis by HPLC.

All patients and controls voluntarily joined the investigation, which complies with the Helsinki Declaration of 1975.

### IEF/IB/LD PROCEDURE

Each serum sample was partially saturated with iron and analyzed in acrylamide gels containing a gradient of ampholytes as previously described [9]. The carbohydrate composition and iron content of the two CDT diagnostic bands quantitated in our system, described in detail elsewhere, represent disialo- and asialotransferrin isoforms [9, 10]. Gels were electrotransferred onto nylon membranes and incubated with rabbit anti-human transferrin antibodies, followed by incubation with an alkaline phosphatase–anti-IgG conjugate. The absorbances of the bands separated by IEF/IB were determined by laser scan densitometry. CDT quantification, expressed as a ratio of CDT to fully sialylated transferrin, was determined by measuring the ratio of the absorbances of the CDT bands vs those of the fully sialylated transferrin bands. This absorbance ratio (calculated for each specimen) was then divided by the absorbance ratio obtained for the strong-positive control in each gel, which served also as a calibrator, multiplied by 100, and expressed in densitometry units (DU). CDT values represent the mean of duplicates. Three controls were run in each gel: a nondrinker woman (CDT−) and two male alcohol abusers: a weak CDT+ and a strong CDT+. Intra- and interassay variations for the weak CDT+ control are 11% and 16%, respectively.

The cutoff limit was established by an analysis of 100 control individuals (50 women and 50 men) with a reported alcohol consumption of <40 g/week; the mean value plus 2 SD was 7.3 DU for women and men combined [10]. Thus, the cutoff was set at 7 DU, and CDT values between 7 and 10 DU, i.e., 2 SD and 3 SD from the mean of the control group, constitute the indeterminate range. When IEF/IB/LD is used in the clinical laboratory, repeat testing is recommended for all those serum specimens with CDT values within this latter range.

### %CDT TIA

The Axis %CDT TIA is a multistep procedure based on microcolumn separation followed by a TIA. Serum transferrin is saturated with iron by mixing 100 μL of serum with 500 μL of an aqueous solution containing 10 mmol/L Bis-Tris [bis(2-hydroxyethyl)aminotris(hydroxymethyl) methane], 0.8 mmol/L Tris, 0.15 mmol/L FeCl₃, 0.15 mmol/L sodium citrate, 0.4 mmol/L maleic acid, 0.5 mL/L Tween 20, and 3.1 mmol/L sodium azide (Solution 1), pH 7.0. We measured the CDT concentration in 500 μL of this master mixture, and we determined the concentration of serum transferrin with 50 μL.

CDT concentration was measured by passing 500 μL of master mixture through a column of 7 mm (i.d.) containing 0.5 mL of Poros HQ10 (Perseptive Biosystems, Framingham, MA). Thereafter, 1.0 mL of Solution 2 (elution buffer), adjusted to pH 6.0 with 1 mol/L HCl and 5 mmol/L NaCl, was added, and the eluate was discarded. Another 2 mL of Solution 2 was added, and the eluate was collected to determine the concentration of CDT (asialo-, monosialo-, disialo-, and 50% of the trisialotransferrins) eluted from the column. In minicolumns, a complete baseline separation between the isotransferrins is difficult to obtain. Including all trisialotransferrins would cause a contamination of tetrasialotransferrins that should be avoided in the eluate. The 50% trisialotransferrin value, calculated through calibration by %CDT HPLC, is therefore a compromise between the clinically ideal 100% trisialotransferrin fraction and avoidance of tetrasialotransferrins in the eluate.

To each well of a microtiter plate was added 200 μL of the eluates from the minicolumn, and the absorbance was determined spectrophotometrically at 405 nm in a microtiter plate reader. A polyclonal anti-human transferrin antibody solution (100 μL; 1 g/L) and 60 g/L polyethylene glycol in 300 mmol/L Tris, pH 7.4, were added to
The concentration of serum transferrin was determined by diluting 50 μL of master mixture with 2 mL of Solution 2, and the absorbance was measured at 405 nm as specified for CDT, before and after addition of the polyclonal anti-transferrin antibody. After the difference in absorbances in the same calibration curve was interpolated, the %CDT:transferrin ratio was calculated for each serum sample in single determinations; the upper reference limit value was 6% for both women and men. Intra- and interassay variations are 4% and 5%, respectively. The reference range was established by CDT evaluations in 43 total abstainers, members of a Norwegian anti-drinking organization, ages 30 to 82 years, and 108 social drinkers, healthy individuals of both sexes, ages 18 to 70 years, consuming an average of <40 g of alcohol daily according to an interview at the time of blood sampling. All total abstainers showed CDT values <5%, whereas 4 of 108 social drinkers showed CDT values >6%. Thus, the upper reference limit was defined as 5% and 6% for total abstainers and social drinkers, respectively.

HPLC ANALYSIS
Analytical HPLC separation was performed at room temperature in a standard HPLC system (Pharmacia Biotech, Uppsala, Sweden) by a procedure similar to the one described by Jeppsson et al. [11]. A Pharmacia HR 5/5 column is high-flow-packed (7–9 mL/min) with Porus HQ10 particles (Perseptive Biosystems) to a gel height of 60 mm. Serum specimens (150 μL) were mixed with the same Fe(III)-Tris-maleic-citrate solution (Solution 1, 30 μL, pH 7) as described for the %CDT TIA, and the samples were incubated for 30 min at room temperature. After incubation, 1.6 μL of dextran sulfate (100 g/L in water) and 7.5 μL of CaCl2 (147 g/L in water) were added to precipitate lipoproteins. The samples were refrigerated for 1 h at 4–6 °C before removal of the precipitated lipoproteins by centrifugation (5000 g) for 15 min at 4–6 °C. The clear supernates (130 g) were diluted 19.5-fold with water and, after a 10-min delay, filtered through a syringe filter (0.22 μm pore size) before injection onto the chromatographic column. Sample storage at 4–6 °C overnight before HPLC analysis causes no detectable alteration of CDT content.

The isoforms of transferrin were separated in a two-buffer system with a multistep salt gradient elution at a flow rate of 2.0 mL/min. Mobile phase A is 20 mmol/L Bis-Tris buffer (Fluka Biochemica, Buchs, Switzerland), pH 6.20. Mobile phase B is 20 mmol/L Bis-Tris buffer, pH 5.60, containing 17.5 g of NaCl per liter (300 mmol/L). Regeneration/cleaning solution consists of equal volumes of methanol and 1 mol/L HCl. Injection of 2 mL of sample volume was optimal with respect to eluant dilution, resolution, and signal intensity.

Peak evaluations in the chromatogram were performed with a Nelson data module. The total transferrin concentration is calculated by baseline integration of all transferrin isoforms; the %CDT concentration is expressed as a relative amount (%) to total transferrin by peak integration calculated for the mono-, di-, and 50% of the trisialotransferrins. Intra- and interassay variation is <5%. The ionic strength of the buffer used in the minicolumn assay was calibrated until the correlation between the HPLC and TIA methods was at maximum. The upper reference limit of 6% CDT was defined as described for the %CDT TIA.

STATISTICAL ANALYSIS
The intraassay variation was calculated by assaying the 3 control specimens, CDT-negative, CDT weak-positive, and CDT strong-positive sera, 15 times each in the same run. The interassay variation was calculated by assaying the same 3 control sera 15 times each in different runs.

We compared the results of the three assays according to the nonparametric Spearman’s correlation coefficient. Diagnostic test performance was evaluated by ROC curve analysis [12], and the area under the ROC curve was calculated by the method of Hanley and McNeil [13] with True Epistat™ software (Epistat Services, Richardson, TX).

Results
CHARACTERIZATION OF CDT ELUATES
The CDT eluted from the ion-exchange minicolumns of the %CDT TIA was analyzed by isoelectric focusing-immunofixation (Fig. 1). The 4 bands present in the alcohol abuser’s sera (lane d) represent pentasialo- (band 6), tetrasialo- (band 5), trisialo- (band 4), and disialo- (band 3) transferrins. After CDT enrichment by minicol-

![Fig. 1. Characterization of CDT eluates.](https://academic.oup.com/clinchem/article-abstract/43/6/983/5640703)
umns, pentasialo- and tetrasialotransferrin bands disappeared, whereas trisialo- and disialotransferrin bands were concentrated in the eluate (lane c). The sera from the control nonabuser (lane b) showed a main band corresponding to tetrasialotransferrin before passing through the minicolumn. After separation by chromatography through a minicolumn (lane a), this specimen shows trisialylated and disialylated isoforms representing <6% of total serum transferrin. This experiment shows the effect of the minicolumns used in the %CDT TIA as a tool for separation of CDT from fully sialylated transferrin isoforms in the alcohol abuser’s sera.

CORRELATION BETWEEN THE CDT TESTS
The correlation between IEF/IB/LD and %CDT TIA ($r = 0.71$, $P < 0.0001$) (Fig. 2, left) was similar to the correlation between IEF/IB/LD and % CDT HPLC ($r = 0.78$, $P < 0.0001$) (Fig. 2, middle). This similarity was expected because all three tests measure relative CDT concentrations rather than absolute CDT values circulating in the blood. The correlation between %CD TIA and %CDT HPLC ($r = 0.88$, $P < 0.0001$) (Fig. 2, right) was the highest among all three procedures because HPLC is not only the confirmatory method but also the calibrator for the turbidimetric immunoassay. In serum samples from two alcohol abusers ingesting >100 g/day, %CDT TIA and %CDT HPLC rendered values slightly above the cutoff (6% CDT), whereas IEF/IB/LD rendered a false-negative CDT result. Another alcohol abuser was CDT-positive by IEF/IB/LD only. Serum specimens derived from three social drinkers tested CDT-positive by IEF/IB/LD, one of whom was also CDT-positive by %CDT TIA but CDT-negative by HPLC. Two African American social drinkers who are carriers of transferrin genetic D variants and three alcohol abusers tested CDT-negative in all three tests.

SPECIFICITY AND SENSITIVITY
The individual CDT values in patients and apparently healthy controls is shown in dot plots (Fig. 3). The results of the Axis %CDT TIA show CDT values below the cutoff (6%) for 8 of 8 total abstainers, 7 of 7 pregnant women and 32 of 33 social drinkers, whereas CDT-positive results were obtained for 26 of 30 alcohol abusers (Fig. 3, top). The sensitivity and specificity within this population were 87% (26 of 30) and 98% (47 of 48), respectively.

The results of the IEF/IB/LD in this same population are CDT-negative results (<7 DU) in 8 of 8 total abstainers, 7 of 7 pregnant women, and 30 of 33 social drinkers, whereas CDT-positive results were obtained in 25 of 30 alcohol abusers (Fig. 3, middle). The sensitivity and specificity within this population were 83% (25 of 30) and 94% (45 of 48), respectively.

%CDT HPLC resulted in CDT values below the cutoff established at 6% for 7 of 7 total abstainers, 7 of 7 pregnant women, and 32 of 32 social drinkers, whereas CDT-positive values were found in 26 of 30 alcohol abusers (Fig. 3, bottom). At a specificity of 100% (46 of 46), the sensitivity of %CDT-HPLC was 87% (26 of 30). The resemblance of the data between both Axis procedures reflects their high correlation coefficient.

ROC ANALYSIS: TEST COMPARISON
The diagnostic efficacy of IEF/IB/LD, %CDT TIA, and %CDT HPLC, defined as the ability to obtain a CDT-positive result when heavy alcohol consumption truly exists (sensitivity or true-positive rate) and to give CDT-negative results when heavy alcohol consumption does not occur (specificity or true-negative rate), are compared in ROC curves (Fig. 4). ROC analysis is a statistical tool that shows sensitivities and specificities for all possible cutoffs in these three tests. At 100% specificity (0% false-positive rate), the sensitivity of both Axis procedures is >87%, whereas the sensitivity of IEF/IB/LD is <50%. This improved clinical utility is shown by a ROC curve that forms an approximate right angle at the upper left side of the ROC plot. The areas under the ROC curves are 0.89, 0.93, and 0.96 for IEF/IB/LD, %CDT HPLC, and %CDT TIA, respectively.

PREDICTIVE VALUES
The diagnostic accuracy for all three CDT procedures was analyzed by means of their predictive values, assuming a prevalence of 7% of alcohol abuse in the US population [14]. The identification of true alcohol abusers among all CDT-positive individuals [positive predictive value (PPV)] as well as predictions for bona fide nondrinkers among all CDT-negative individuals [negative predictive...
value (NPV) was calculated with the cutoff values established for each test (Table 1). All three CDT procedures show the same optimal NPV (0.98); i.e., 98% of individuals with a CDT-negative result are nondrinkers. The PPV varied from 0.5 for the IEF/IB/LD to 0.75 for the %CDT TIA and 1.0 for the %CDT HPLC. Therefore, only one-half (50%) of individuals with positive CDT results by IEF/IB/LD correspond to true alcohol abusers, and one-half (50%) represent false-positives compared with %CDT TIA and %CDT HPLC with 75% and 100% accuracy rates, respectively.

Discussion

%CDT TIA and %CDT HPLC both include 50% of the trisialotransferrins in their quantitation schemes and showed an overall improved performance compared with IEF/IB/LD, which measures only disialo- and asialotransferrins in serum. The %CDT TIA procedure described here is the first CDT test adapted for semiautomation and simultaneously calibrated with a confirmatory HPLC method. Consequently, similar results are obtained on an array of turbidimetric instruments, e.g., the Cobas Fara, Cobas Mira S, Behring BN100, BNA, BNII, Hitachi 707, 911, IL 900, IL 1800, and Kone Optima. In addition to automation, the %CDT TIA has a turnaround time of 3.5 h and is nonradioactive and cost effective. Even though each specimen requires two measurements, the values of CDT and total transferrin are both interpolated into the same calibration curve when %CDT is calculated. Its precision is low enough to allow for single determinations of CDT and total transferrin in each specimen.

The pioneer IEF/IB/LD was the first CDT procedure to measure CDT as a ratio of total serum transferrin; CDTect measures absolute values of circulating CDT. CDT:transferrin ratios provide increased diagnostic accuracy rather

Fig. 3. Distribution of CDT values in alcohol abusers and control nonabusers as measured by %CDT TIA (top), IEF/IB/LD (middle), %CDT HPLC (bottom).

Fig. 4. ROC curves for the three methods for CDT quantitation in alcohol abuse.
than absolute CDT values because individuals with high or low serum transferrin concentrations will render a false-positive or a false-negative result, respectively, with CDTect. Like IEF/IB/LD, %CDT TIA and %CDT HPLC measure CDT as fractions of total serum transferrin.

An ROC curve is the best way to compare the diagnostic performance of different procedures because it shows sensitivities and specificities for all possible cutoffs in a test. In this study, the dot plots illustrate the distribution of individual values obtained by each procedure, whereas the ROC plot compares the diagnostic performance of the three CDT tests. Both Axis procedures perform better diagnostically than the IEF/IB/LD, which measures asialo- and disialotransferrin, but not the trisialotransferrin isoforms. A similar ROC curve was obtained for the IEF/IB/LD procedure as for the CDTect, a finding that supports the reliability of the former as a comparison method for the validation of new CDT tests [4].

Clinical outcome decides whether the test results would assist in improving the patient’s health at an affordable cost and includes the efficacy variables of the test: sensitivity, specificity, PPV, and NPV [15, 16].

Sensitivity answers the question: "If the patient has the disease, how likely is he to have a positive test?" Specificity answers the question: “If the patient does not have the disease, how likely is he to have a negative test?” A clinician’s question is, “If the patient has a positive test, how likely is he to have the disease?” or “If the patient has a negative test, how likely is he not to have the disease?” [17].

In family practice, most conditions, including alcohol abuse, are of low prevalence. The PPV will be lower and the NPV will be higher in populations with low prevalence for a disorder [18]. Because the prevalence of alcohol abuse is 5–7% in the US, positive CDT results are more likely to represent false positives than true positives. CDT procedures like IEF/IB/LD show a PPV of 50% in the general population; i.e., only one of two CDT-positive results reflect identification of a true alcohol abuser. The clinical utility of this pioneer CDT procedure in detecting alcohol abuse in the general population would be much improved if the assay was used as a reflex test after screening with more-conventional diagnostic tests [19]. Thus, with the same sensitivity and specificity rates described for the IEF/IB/LD, the PPV increases from 0.5 to 0.8 in a preselected population where the prevalence of alcohol abuse is 25%.

Previous results have been discouraging for CDT used to screen the general population [6–8, 20]. The Svalbard study defined heavy drinkers according to the amount of alcohol ingested by the 95th percentile of the population examined, e.g., 52 g/day for men [6]. However, the optimal diagnostic performance of CDT has been reported for an alcohol consumption of at least 60 g/day for 7–10 consecutive days [1–5]. The Copenhagen study used a cutoff point for CDT that maximized sensitivity (82%) rather than specificity (77%) [21]. Considering the low prevalence of alcohol abuse in the general population, at a specificity of 77% the PPV is <0.2. Bell et al. [8] reported a sensitivity of 69% at 92% specificity for 502 patients admitted consecutively to a medical department, a value similar to that generally described for CDTect in individuals ingesting >50 g of alcohol per day.

Newly emerging procedures like %CDT TIA and %CDT HPLC, however, provide increased diagnostic performance because of improved sensitivity and specificity [22]. Thus, the PPV improved from 0.5 for IEF/IB/LD to 0.75 with these new, semiautomated CDT tests; three of four alcohol abusers like the ones defined in this study (group 4) could now be accurately identified in a clinical practice. CDT procedures like %CDT TIA and %CDT HPLC show the benefits of improved diagnostic performance and cost-effective automation, with the potential for widespread use of this marker in several clinical settings. Whether the inclusion of the trisialotransferrins results in improved efficacy of the test awaits further analysis of the carbohydrate composition of CDT.

### Table 1. Predictive values for three CDT tests at a 7% prevalence of alcohol abuse.

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<th>Alcohol abuser</th>
<th>Total no. of subjects</th>
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<td>(6/12)</td>
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