Two Methods for Measuring Carbohydrate-Deficient Transferrin in Inpatient Alcoholics and Healthy Controls Compared

Raymond Anton¹ and Pamela Bean²,³

Carbohydrate-deficient transferrins (CDTs), naturally occurring glycosylated transferrin proteins, are reported to be increased in the serum of individuals who consume large quantities of alcohol (ethanol). We compared two methods for the separation and quantification of CDT, using the same alcohol-dependent patients and age-, gender-, and race-matched controls as sources of samples for both assays. There was good correlation (r = 0.89) between the microcolumn anion-exchange chromatography/RIA (MAEC/RIA) procedure and the isoelectric focusing, immunoblotting, and laser densitometry (IEF/IB/LD) procedure. Receiver operating characteristic analysis suggested that the IEF/IB/LD procedure would perform slightly better than MAEC/RIA for the overall population. However, both assays were more sensitive for the detection of heavy alcohol consumption in men, compared with women. Alcohol consumption in the week prior to CDT measurement correlated only weakly with the concentrations measured with either assay.

Indexing Terms: alcoholism/receiver operating characteristic curve/laser densitometry/immunoblotting/chromatography, ion exchange/sialic acid/isolectric focusing

Individuals who abuse alcohol (ethanol) are notorious for underreporting the amount they consume, even in situations directly related to the evaluation of their health. Treatment outcome studies show that alcohol-dependent individuals err in reporting their alcohol use so often that independent corroboration of alcohol intake is necessary. Hence, there is a need for a sensitive and specific biological test for heavy alcohol consumption (1, 2). Although liver enzyme tests are used because of relative ease of measurement and availability, even the most sensitive and specific of these tests, that for γ-glutamyltransferase (GGT), is only 40–60% (1, 3, 4) sensitive and 80% specific for detection of continuous high alcohol consumption. In the presence of other liver pathology, the specific association of high GGT concentrations with recent alcohol consumption is even less useful.

A new method for detecting heavy alcohol consump-

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⁴ Nonstandard abbreviations: GGT, γ-glutamyltransferase; CDT, carbohydrate-deficient transferrin; MAEC, microcolumn anion-exchange chromatography; IEF/IB/LD, isoelectric focusing/immunoblotting/laser densitometry; ROC, receiver operating characteristic; DU, densitometry units; and OD, optical density.

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tion has generated much interest (3–5). Carbohydrate-deficient transferrins (CDTs) are produced in greater quantities than usual during periods of heavy alcohol intake, e.g., 50–80 g per day over several weeks. Decreased glycosylation of the transferrin protein before hepatocellular release is the most likely mechanism for the production of these transferrin variants. After separation from the larger amounts of normal transferrin on the basis of different charge characteristics, CDT can be quantitated by means of antibody-based techniques. The two major methods developed for this are microcolumn anion-exchange chromatography (MAEC) with quantitation by RIA (MAEC-RIA) and isoelectric focusing/immunoblotting (IEF/IB) with quantitation by laser densitometry (LD). The former is utilized extensively in Scandinavia and Western Europe (5), whereas investigators in the US report that IEF is superior, especially in clinical populations in which nonalcoholic liver disease is prevalent (6).

The sensitivity and specificity of these methods are affected by a number of variables, including appropriateness of the control or normative population, gender, frequency and quantity of alcohol use, timing of blood sampling after cessation of use, and possibly non-alcohol-related illnesses such as primary biliary cirrhosis, and genetic makeup. Other factors, such as separation efficiency, analytical sensitivity, and iron content in normal and CDT isoforms, are assay-specific.

Here we compared these two dissimilar techniques, IEF/IB/LD and MAEC/RIA, for detection of CDT in samples from well-characterized alcohol-dependent patients and controls with minimum alcohol consumption.

Patients and Methods

Patients and Controls

Fifty-nine patients, who voluntarily entered a substance-abuse detoxification unit and were dependent on alcohol during the period immediately preceding admission, signed Institutional Review Board for Human Research-approved informed consent forms. The patients were interviewed 48–72 h after admission, when the disruptive cognitive effects of acute alcohol intoxication and withdrawal had diminished sufficiently to allow recall. Daily alcohol consumption in the month before admission was documented with a modified timeline follow-back procedure (7). Serum, generally obtained within 48 h (86% of patients) of the last alcoholic drink (range 8–96 h), was placed in polypropylene tubes and stored at −70°C until assayed. Control subjects, generally medical center employees or students, were interviewed over a period of time corresponding to the collection of patients' samples. Sixty-one control subjects,
selected to match each patient in age (within 5 years), race, and gender, filled out questionnaires and were interviewed by a research psychiatrist about general health, alcohol and substance ingestion, medication use, and other pertinent clinical issues. Control subjects uniformly scored below the cutoff for alcohol-related problems on the Short Self-Rated Michigan Alcohol Screening Test (the overwhelming majority scored zero), and none met alcohol abuse or dependence criteria according to DSM-III-R. Alcohol intake, quantified over the month prior to the interview with the timeline follow-back procedure, was <15 g of alcohol per day; the majority were either minimal alcohol users or complete abstainers. Serum samples from each patient and matched control were assayed under the same conditions, usually within 3 months of collection.

MAEC/RIA

We thawed one tube of serum from each subject and subjected it to the CDTect procedure (Kabi-Pharmacia, Uppsala, Sweden) as detailed by Stibler et al. (5). In brief, 50 μL of serum was preincubated with 200 μL of ferric citrate and 1 mL of elution buffer. Lyophilized microcolumns were reconstituted and equilibrated with 2 mL of elution buffer. We added 500 μL of preincubated sample to each microcolumn. The eluate, which reportedly contains isoforms of transferrin with pls >5.7, was then subjected to RIA for quantitation. We performed a double-antibody RIA with rabbit anti-human transferrin antibody to bind CDT and with sheep anti-rabbit antibody to precipitate the complex. After centrifugation, we counted the radioactivity of the precipitate in a Packard (Meriden, CT) gamma counter. The amount of CDT in duplicate samples was calculated from a five-point calibration curve derived from the displacement of [125I]CDT from the antibody by known amounts of human transferrin. A new calibration curve was prepared for each assay.

IEF/IB/LD

Single-blinded samples were identified by number only and assayed by IEF/IB/LD without knowledge of patient or control status. IEF/IB/LD was performed as described (8). Briefly, sera were partially saturated with iron by incubation in a buffered iron-containing solution (0.2 mmol/L FeCl₃, 12 mmol/L sodium phosphate, 5 mmol/L sodium citrate, pH 7.2) at 37°C for 90 min. We carried out IEF analysis of sera in 5% polyacrylamide gels containing a pH 4–8 gradient of ampholytes (LKB Pharmacia, Piscataway, NJ). IEF gels were electro-transferred to nylon membranes (Millipore, San Francisco, CA) by standard procedures. Incubation with rabbit anti-human transferrin antibodies (1:1000 dilution; Dako, Carpinteria, CA) was followed by incubation with alkaline phosphatase-conjugated anti-rabbit IgG (Tago Immunologicals, Burlingame, CA) at room temperature for 60 min. Color was developed with the Bio-Rad alkaline phosphatase kit (Bio-Rad, Richmond, CA). We included three control sera in each immunoblot: negative, weakly positive, and strongly positive. The optical densities (OD) of the bands separated by IEF/IB were determined with a densitometer (Molecular Dynamics, Sunnyvale, CA) that has a laser scanner and a light-integrated cylinder for quantitation.

Immunoblots were scanned with a setup of 100-μm pixels with 12-bit data size (= 20K disk space per cm²). Each lane (specimen) in the blot was enclosed by a grid made up of two rows and one column. The upper row of the grid enclosed the two CDT diagnostic isoforms (bands 8 and 9), and the lower row of the grid enclosed all other transferrin isoforms (bands 1 to 7). Using volume integration, we determined the sum of OD values within the grid, then quantified the CDT by a three-step process: (a) For each specimen, the ratio of the OD of the criteria bands (upper row of the grid) to the OD of the other transferrin bands (lower row of the grid) was calculated; (b) the OD ratio calculated for each specimen was divided by the OD ratio obtained for the strongly positive control in each gel; and (c) the value for each specimen was multiplied by 100 and expressed in densitometry units (DU).

Statistical Analysis

We compared the results of the two assays by using Pearson's correlation coefficient. We compared the mean CDT values obtained by IEF/IB/LD for men and women by Student's t-test. Diagnostic test performance was evaluated by receiver operating characteristic (ROC) analysis (9), and the area under the ROC curve was calculated by the method of Hanley and McNeil (10). The relation of the reported alcohol intake in the week before admission to the hospital to the measured CDT concentrations was evaluated by linear regression analysis.

Results

The age, race, and gender of patients and controls were very similar (Table 1). Although the range of alcohol intake was wide during the 2 weeks before hospital admission and blood testing, all patients consumed >60 g of alcohol per day on average. No control subject consumed >15 g per day on average; the majority of controls did not drink any alcohol in the month before sample collection.

Table 1. Demographic data on heavy-alcohol-consuming patients presenting for detoxification and the matched control population.

<table>
<thead>
<tr>
<th></th>
<th>Patients</th>
<th>Controls</th>
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<tbody>
<tr>
<td>n</td>
<td>59</td>
<td>61</td>
</tr>
<tr>
<td>Age, years</td>
<td>40 ± 10</td>
<td>39 ± 10</td>
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<tr>
<td>Gender</td>
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<td>Men</td>
<td>41</td>
<td>40</td>
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<tr>
<td>Women</td>
<td>18</td>
<td>21</td>
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<tr>
<td>Race</td>
<td></td>
<td></td>
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<tr>
<td>Caucasian</td>
<td>48</td>
<td>50</td>
</tr>
<tr>
<td>African-American</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>Average alcohol consumption, g/day</td>
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<td></td>
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<tr>
<td>Men</td>
<td>258 ± 174</td>
<td>&lt;15</td>
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<tr>
<td>Women</td>
<td>138 ± 84</td>
<td>&lt;15</td>
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Standards and patients' and control samples were assayed in duplicate; the mean was used for all calculations and reported values. The intra- and interassay CVs were 7.6% and 14% for MAEC/RIA and 11% and 21% for IEF/IB/LD, respectively. The results obtained with MAEC/RIA and IEF/IB/LD correlated well ($r = 0.89; P < 0.0001$) for patients and controls (Fig. 1).

The diagnostic performance of these two procedures, defined as the ability to detect heavy alcohol consumption when it truly exists (sensitivity or true-positive rate) and to give normal results when heavy alcohol consumption does not occur (specificity or true-negative rate), was evaluated by ROC curve analysis with combined gender data (Fig. 2). Whereas the ROC curves for both procedures were similar, the area under the IEF/IB/LD curve (0.88) was significantly ($P = 0.01$) larger than the area under the MAEC/RIA curve (0.73) as calculated by the method of Hanley and McNeil (10).

Because at specificities <80% the two ROC curves were essentially identical, this difference appears to be accounted for in the higher specificity areas of the ROC plot. At specificities >80%, IEF/IB/LD offers slightly greater detectibility of the alcohol abuse condition than does MAEC/RIA; i.e., at 90% specificity (10% false-positive rate), the sensitivity obtained by IEF/IB/LD was 76%, compared with 69% obtained by MAEC/RIA.

The distribution of CDT values in the patient population and normal controls, as measured by these methods, is shown in dot plots (Fig. 3). Since previous data showed a gender difference in the distribution of CDT concentrations (4, 11), the data for men and women are presented separately. In this study, the mean CDT values as measured by MAEC/RIA were 12 ± 4 units/L and 33 ± 21 units/L for male controls and patients, respectively. The mean CDT values measured by IEF/IB/LD for these groups were 3 ± 2 DU and 22 ± 22 DU, respectively. At 1 SD above the average control value, sensitivities and specificities were comparable for MAEC/RIA and IEF/IB/LD, i.e., 85% and 90% sensitivities and 88% and 85% specificities for the respective assay methods (Table 2). At 2 SD above the average control value, the sensitivity was better for the IEF/IB/LD than for MAEC/RIA, i.e., 85% vs 66%. The specificities at 2 SD were comparable, i.e., 93% for IEF/IB/LD and 98% for MAEC/RIA.

For women, the mean CDT values as determined by MAEC/RIA were 17 ± 5 units/L and 30 ± 17 units/L for controls and patients, respectively. The CDT values determined for these groups by IEF/IB/LD were 5 ± 2 DU and 15 ± 18 DU, respectively. Both methods provided lower sensitivities for women than for men at both 1 and 2 SD above the average control value (Table 2). At 1 SD above the average control value, sensitivity was better for the MAEC/RIA than for IEF/IB/LD, whereas the specificities were similar, i.e., 72% and 50% sensitivities and 80% and 86% specificities for the respective assay methods. At 2 SD above the average control value, both assays showed relatively poor sensitivity, i.e., 44% for MAEC/RIA and 33% for IEF/IB/LD, whereas specificity was high for both methods, i.e., 100% for MAEC/RIA and 96% for IEF/IB/LD.

The overall correlation of alcohol intake for all patients (n = 59) in the week before admission and CDT values by either the MAEC/RIA or IEF/IB/LD procedure was low and nonsignificant ($r = 0.03$ and 0.05, respectively). This reflects a marked variation in the amounts of CDT produced by individuals despite similar amounts of daily alcohol consumption. Considering that at very high levels of daily alcohol consumption (>350 g/day) there was less increase in CDT than in many patients who had lower levels of alcohol intake (data not shown), we analyzed only those patients (n = 52) who consumed <350 g of alcohol daily. The correlation of CDT vs daily alcohol consumption in this population was $r = 0.24$ (P
Women a range. Sensitivities techniques concentrations RIA <0.08). Although than 3. Alcohol data h z. 3. I. Distribution after Cootrot and Alcohol N-41 S. 7. a. I- Distribution of their MAEC/RIA was 9). Patients N=40 Ur! = 0.18 patients who whose measurement was taken sooner. The sensitivity for detection of heavy alcohol consumption by two methodologies of CDT measurement with different cutoff points.

<table>
<thead>
<tr>
<th></th>
<th>Men</th>
<th>Women</th>
<th>Men</th>
<th>Women</th>
</tr>
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<tbody>
<tr>
<td>MAEC cutoff, units/L</td>
<td>16</td>
<td>20</td>
<td>22</td>
<td>27</td>
</tr>
<tr>
<td>Sensitivity, %</td>
<td>85</td>
<td>66</td>
<td>72</td>
<td>44</td>
</tr>
<tr>
<td>Specificity, %</td>
<td>88</td>
<td>98</td>
<td>80</td>
<td>100</td>
</tr>
<tr>
<td>IEF/IB cutoff, DU</td>
<td>5.0</td>
<td>7.0</td>
<td>7.6</td>
<td>10.0</td>
</tr>
<tr>
<td>Sensitivity, %</td>
<td>90</td>
<td>85</td>
<td>50</td>
<td>33</td>
</tr>
<tr>
<td>Specificity, %</td>
<td>85</td>
<td>93</td>
<td>86</td>
<td>95</td>
</tr>
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Discussion

Although opinion is converging that CDT measurement is the most sensitive and specific test for detection of heavy alcohol consumption (3, 4), direct comparisons of IEF/IB/LD and MAEC/RIA have not been reported. In this study we observed a significant correlation between the values obtained by these two dissimilar techniques. The ROC curves indicate great accuracy for both these techniques to discriminate between drinkers and non-drinkers. The area under the curve is greater for IEF/IB/LD than for MAEC/RIA; at 85–95% specificity, IEF/IB/LD appears to be somewhat more sensitive. Small changes in the ROC curve can lead to statistically significant differences in the areas measured, but with little clinical significance when assays correlate closely (9).

Even though Xin et al. (12) found a high correlation (r = 0.98) for CDT measured by an older MAEC method and their IEF/IB, the improved MAEC method (6) correlated less well (r = 0.60). In our study, the correlation (r = 0.89) of the newer MAEC/RIA technique with our IEF/IB/LD approach is remarkably better. However, patients with liver pathology were not included in our analysis, whereas the group of individuals with liver pathology is the focus of the studies of Xin et al. Nevertheless, there is no reason to believe that these techniques perform differently in separating and quantitating CDT in different patient populations.

Sensitivities for detecting heavy alcohol consumption by increased CDT concentrations range from 60% to 100%, with the average estimated to be 82% for 2500 patients (4). In our study, sensitivities for the MAEC/RIA and IEF/IB/LD methods fell within the reported range. As expected, sensitivity decreased as specificity increased. Although sensitivity at both 1 and 2 SD above the average control value was somewhat better for the IEF/IB/LD than the MAEC/RIA method for detecting CDT in men, either method provided useful information for the detection of alcohol abuse if proper cutoff values were set.

The correlation between CDT by the MAEC/RIA (r = 0.24) and the IEF/IB/LD (r = 0.18) methods and the amount of alcohol consumed in the week prior to blood sampling was similar to the correlation of 0.36 reported by Stibler et al. (13) for patients consuming <350 g of alcohol/day. This implies that the amount of alcohol consumed can only account for <10% of the overall variance in the amount of CDT produced. Interindividual differences in the sensitivity of the hepatocyte to

Fig. 3. Distribution of CDT values in (A) male and (B) female controls and alcohol abusers as measured by two procedures.
produce abnormal transferrin might exist so that once a threshold amount of alcohol consumption is reached, increasing amounts add very little to abnormal transferrin production. In addition, lower amounts of transferrin, and by extrapolation CDT, are produced by the liver at very high levels of alcohol consumption (14), possibly indicating cellular loss and a cirrhotic process. Our preliminary results showed low CDT values for six patients consuming >350 g of alcohol daily.

One unique feature of the IEF/IB/LD technique is its ability to discriminate between transferrin isoforms deficient in sialic acid residues (true CDT) and normal transferrin lacking iron (pseudo-CDT). The genetic variants of transferrin can also be identified by IEF/IB/LD, making it feasible to rule out false-positive results due to genetic polymorphism (P. Bean, manuscript in preparation). The MAEC/RIA method is particularly applicable to situations in which exact quantitation of CDT concentrations is important, e.g., monitoring scores for individual patients during longitudinal treatment or comparing specific concentrations in pregnant women, children, or the elderly.

We have examined the utility of CDT measurement in discriminating between a heavily drinking clinical population and controls. Extrapolation of sensitivity results to nonclinical populations can only be done in an empirical fashion. For instance, CDT has been found to be much less sensitive (20–25%) as a screening tool for heavy alcohol consumption in university students (15) and for community health screening (16).

In summary, we have presented data that support the utility of CDT determination for the sensitive and specific detection of heavy alcohol consumption in an American patient population. Both procedures for CDT measurement provided strong diagnostic accuracy for men. For women, however, additional studies should consider the variability in transferrin glycosylation induced by factors such as hormonal and iron status (17). The highly significant correlation (r = 0.89) between these methodologies indicates that both measure transferrin isoforms that vary from individual to individual and with alcohol intake. These studies should be extended to include patients with psychiatric disorders, medical illnesses, and abuse of other substances.

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References