Comparison of HPLC and Small Column (CDTect) Methods for Disialotransferrin

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Background: Current methods for determination of carbohydrate-deficient transferrin (CDT) are based on separation of the CDT fraction by ion-exchange chromatography on minicolumns and quantification by immunoassay. Alternatively, the transferrin isoforms can be separated by HPLC anion-exchange chromatography and quantified by absorbance. This method has been reported to improve the validity of CDT as a marker of chronic alcohol abuse.

Methods: HPLC on either MonoQ or ResourceQ anion-exchange columns was used to separate and quantify isoforms of transferrin with detection at 460 nm. The result was expressed as the percentage of the disialo form (pI 5.7) of total transferrin (DST). The commercial CDTectTM assay was used as a comparison method. Serum samples from nondrinkers (n = 57), moderate drinkers (n = 77), and heavy drinkers (n = 139) were analyzed.

Results: In ROC analysis for differentiation between moderate and heavy drinkers, the area under the curve (AUC) for the HPLC method was 0.87 (95% confidence interval, 0.81–0.93), whereas that for CDTect was 0.72 (95% confidence interval, 0.64–0.80). At 90% specificity, the sensitivity of DST was 63% (95% confidence interval, 53–73%) compared with 33% (22–44%) for CDT. The reference interval of the HPLC method was 0.68–1.7%.

Conclusions: The HPLC anion-exchange method for quantification of CDT provides substantially better separation between moderate and heavy drinkers than the CDTect method.

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Materials and Methods

REAGENTS

All chemicals were of analytical reagent grade. CDTect reagent sets were purchased from Bio-Rad (Axis Biologicals ASA).

APPARATUS

For HPLC analyses, we used a chromatographic system consisting of a Varian 9010 HPLC pump (Varian Associ-
Sample Treatment for HPLC
One mL of fresh serum or serum that had been stored at −20 °C was saturated with iron by the addition of 25 μL of 0.5 mol/L NaHCO₃ and 18 μL of 10 mmol/L FeCl₃. After the sample was mixed and incubated for 1 h at room temperature, the lipoproteins were precipitated by the addition of 10 μL of 100 g/L dextran sulfate (Sigma Chemical Co.) and 50 μL of 1 mol/L CaCl₂. This mixture was kept at 4 °C for 30–60 min and then centrifuged at 10 000 g for 10 min. The supernatant was diluted fivefold with water, filtered through a 0.45 μm filter unit.

HPLC Conditions
The pretreated sample (150 μL) was injected into the column. The transferrin isoforms were separated by a salt gradient. Buffer A was 20 mmol/L Bis-Tris buffer, pH 6.2; buffer B was buffer A plus 0.35 mol/L NaCl, pH 6.2. Solution C, consisting of 0.5 mol/L NaCl, was used for column regeneration. Before use, all solutions were filtered through a 0.45 μm filter. Separation was achieved by gradient elution (Table 1) at room temperature with a flow rate of 1 mL/min. The detection wavelength was 460 nm. Integration was performed in the horizontal baseline mode. The area for each individual isoform was reported as the percentage of the total area of transferrin. The chromatographic system is usually loaded with 70–80 columns were used for samples and 2 for controls. Labor costs were estimated according to our local expenses. Reagent cost per assay was calculated on the basis of single assays, with six calibrators (for CDTect) and two controls. Labor costs were estimated according to our local expenses. The cost of the reagent set (US$400) was calculated on the basis of optimal usage, i.e., 48 minicolumns were used for samples and 2 for controls. Labor costs were estimated according to our local expenses. Reagent cost per assay was calculated on the basis of single assays, with six calibrators (for CDTect) and two controls in each HPLC run.

Results
HPLC Separation of Transferrin Isoforms
Typical chromatograms of serum samples are shown in Fig. 1. Quantification relies on the selective absorbance of the iron-transferrin complex at 460 nm. Asialo-, disialo-, trisialo-, tetrasialo-, and pentasialotransferrin were separated as distinct peaks. Disialotransferrin (DST; pI 5.7) eluted as a separate peak at 11.7 min. The variation in retention time (CV) over 20 runs was 0.5%. The majority of the serum samples gave chromatograms similar to that shown in Fig. 1B. Virtually identical separation was
obtained with the ResourceQ column (Fig. 1C). For routine analysis we used the ResourceQ column because it is more economic and the separation of transferrin isoforms is similar to that obtained on the MonoQ. The interassay CVs for two samples (ResourceQ) were 6.5% and 4.3% (1.2% and 3.7% DST, respectively; 15 replicates each) and was 12% for CDTect (23.3 units/L; 15 replicates). The determinations were conducted on serum pools stored in frozen aliquots and thus reflect the entire process.

The selection of HPLC instrumentation is not critical, but the detector needs to be sensitive. For example, the Spectroflow 783 (ABI Analytical) and the HP 1100 (Agilent Technologies) detectors provided reliable results, but some other detectors were less suitable. The gradient used in this study differs slightly from those used in other studies (6, 12), and it is likely that some adjustment must be made when switching to other HPLC systems.

REFERENCE INTERVAL
We analyzed serum samples (n = 165) from apparently healthy blood donors. After we eliminated eight obvious outliers with values from 2.2–3.1%, the proportion of CDT was 0.55–1.9%, the mean (± SD) was 1.13% ± 0.27%, and the median was 1.10%. The upper reference limit, based on the 97.5 percentile, was 1.7%, and the lower limit, based on the 2.5 percentile, was 0.68% for the whole group. The upper reference limit was 1.6% for women (n = 85) and 1.8% for men (n = 80). In women, the mean DST was 1.10% and the median was 1.10%, whereas the mean was 1.18% and the median was 1.10% in men (P = 0.066 between the groups).

CORRELATION OF METHODS
The correlation of the HPLC (y) and the CDTect assay (x) was determined with 189 samples from nondrinkers and moderate and heavy drinkers (Fig. 2). The correlation was: \( y = 0.09x - 0.26; r = 0.72; S_{xy} = 1.3\% \).

With the cutoff of 1.8% for DST and 20 units/L for CDT and by use of an alcohol consumption of 210 g/week as the cutoff for heavy drinking, 52% of the heavy drinkers had increased results by HPLC compared with 49% by the CDTect assay (Fig. 3). However, the specificity of the HPLC method (98%) was substantially higher than that for CDTect (84%). Among the moderate drinkers, 4% had increased results by CDTect and 2.6% by DST. Both methods showed a positive correlation between alcohol consumption and percentage of DST.
consumption and CDT values, but the correlation \( r \) was stronger with the DST method than with the CDTect, 0.54 vs 0.40 (Fig. 3).

At 90% specificity, the sensitivity for DST was 63% (95% confidence interval, 22–44%) compared with 33% (95% confidence interval, 53–73%) for CDT.

ROC analysis showed that the area under curve (AUC) for differentiation between moderate drinkers and heavy drinkers (Fig. 4), was significantly larger \( (P = 0.0002) \) for DST (0.87; 95% confidence interval, 0.81–0.93) than for CDT (0.72; 95% confidence interval, 0.64–0.80). We also determined the ratio of DST to trisialotransferrin by HPLC. The AUC for this ratio was lower (0.81) than that for DST.

Estimated costs for the HPLC assay, assuming 3000 assays/year, were approximately one-half those of the CDTect assay (Table 2). The costs for the HPLC assay would be similar to those of the CDTect assay if we analyzed 1000 samples yearly. Because only 40–60% of the capacity of the HPLC instrumentation is used for the DST assay, the actual costs are ~10% lower. Because of reproducibility problems, we have in practice run duplicates in the CDTect assay; thus the cost of this assay has been approximately double that indicated in Table 2.

Fig. 3. Correlation of alcohol consumption (x axis) and CDT (y axis) in 214 sera from nondrinkers and moderate and heavy drinkers by HPLC (A) and CDTect (B). The dashed lines indicate the upper reference limits. Corresponding regression equations are: (A), \( y = 0.0003x + 1.12 \) \( (r = 0.54) \); (B), \( y = 0.002x + 16.1 \) \( (r = 0.40) \).

Discussion

CDT has become the most important laboratory test for the detection of prolonged heavy alcohol consumption (7). In routine methods, the transferrin isoforms are separated into two fractions by anion-exchange chromatography on microcolumns and the CDT fraction quantified by RIA (CDTect) or immunoturbidimetry (%CDT-TIA; Axis). The fraction referred to as CDT is not precisely defined and contains, in addition to asialo-, monosialo-, and disialotransferrin, variable amounts of trisialotransferrin, whereas part of DST is retained on the column (13). Isoelectric focusing provides good separation of the iso-transferrins, but it requires immunostaining of the separated transferrins before quantification. It is therefore considered too complicated and time-consuming for routine use. The major transferrin isoforms can also be separated by anion-exchange chromatography. We evaluated a slight modification of the HPLC method originally developed by Jeppsson et al. (6), based on anion-exchange chromatography after iron saturation of transferrin in the sample. Detection of the iron-transferrin complex at 460 nm is highly specific, and good separation of the transferrin isoforms is obtained. The chromatogram
is a visible document of successful isotransferrin determination. Common genetic variants of transferrin, which can cause erroneous results in interpretation of the CD-Tect method (13), do not pose a problem. Rare variants that cause problems in minicolumn chromatography have been reported to occur in Caucasians (7, 14). No such variants were detected in the present study, apparently because these variants are rare in the Finnish population.

ROC analysis demonstrated that the HPLC method had a substantially better ability to distinguish between moderate and heavy drinkers than the CD-Tect. The probable explanation for this is the use of the proportion of DST in relation to total transferrin. This approach eliminates the confounding effect of variations in transferrin concentrations. This problem has been eliminated in a recently introduced method in which both of the fractions are measured by immunoassay (%CDT-TIA; Axis). This method has also been reported to have better diagnostic accuracy than the CD-Tect assay in some studies, but not in others (7, 15). The most likely explanation for the superior clinical performance of the DST assay is the good chromatographic separation. Nearly baseline separation between the important isoforms was achieved by HPLC. This is analogous to the improvement in performance when HPLC methods replaced batchwise methods for separation of glycohemoglobins (16).

We also studied whether the ratio of disialo- to trisialotransferrin improved the discriminatory power of the HPLC method, but this was not the case, as evidenced by a smaller AUC. This is in agreement with previous results that indicated that the measurement of trisialotransferrin provides no diagnostic advantage (17, 18).

The reference limits were determined based on samples from apparently healthy blood donors and reflect the general population in Finland. Some of these donors probably consume more alcohol than the moderate drinkers included in the study. We therefore eliminated outliers before calculating the reference interval. With these reference values, only 2.6% of the moderate drinkers had increased DST values, and the highest value was 3.1%.

The upper reference limit for CDT established in this study, 1.7%, is higher than the previously reported value of 1% (12, 19). This difference can be explained by differences in the integration mode of the chromatographic profile. The horizontal baseline integration mode used in our study gives higher results for minor components than the valley-to-valley mode used in a previous study (6). We decided to use this method because it provides more realistic values and is less sensitive to variations in peak separation. Interestingly, we observed lower values in women than in men, although higher mean values (12) and spuriously increased CDT values are often observed in women (20). In spite of several hypotheses (7), the reason for this remains unclear. Because of the stigmatizing effect of an increased marker for alcohol abuse, these falsely increased results are of great concern.

HPLC methods usually are considered more expensive than immunoassays; in clinical chemistry, therefore, they are used mainly for determination of drugs and glycohemoglobin on dedicated instruments. Because higher cost is a common argument against the use of HPLC methods, we estimated the costs of the two methods. This showed that in our laboratory the HPLC method was actually much more cost-effective. This is because we analyze a fairly large number of samples. With 1000 samples/year, the costs of the HPLC and the CD-Tect assays would be similar. However, this calculation does not include expenses for establishing the assay. The costs of setting up an in-house HPLC method may be substantial unless experienced personnel are available.

In conclusion, our results show that the ion-exchange HPLC method is more valid than the CD-Tect method for detection of excessive alcohol use. These results are in line with those of two previous studies (12, 19). In agreement with one of these studies (12), the diagnostic value of the CD-Tect method was so low that its clinical utility must be considered questionable and should probably be replaced by the newer %CDT-TIA assay from the same manufacturer or by HPLC. It remains to be determined which of these assays provides the most accurate clinical information.

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References


