Carbohydrate-Deficient Transferrin Quantified by HPLC to Determine Heavy Consumption of Alcohol

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We developed a new fully automated ion-exchange chromatographic method for quantitating carbohydrate-deficient transferrin (CDT) on a Mono Q™ column. Quantitation relies on the selective absorbance of the iron-transferrin complex at 460 nm. Transferrin isoforms deficient in sialic acid, with pls 5.7 and 5.9, can easily be separated and quantitated as a percentage of the total transferrin. This method has been applied to samples from teetotalers, occasional drinkers, patients with recent heavy alcohol consumption, and patients during detoxification. The sensitivity of the method was 55% in patients reporting 40–70 g daily ethanol consumption and nearly 100% in heavily intoxicated patients (70–500 g daily consumption). The half-life of the dominating pl 5.7 isoform in this group was 9.5 (± 1) days during detoxification. A CDT value > 0.8% is a highly specific marker for alcohol abuse and is greatly superior to other currently available biological markers.

Indexing Terms: ion-exchange chromatography • isoelectric focusing • transferrin isoforms • sialic acid

Alcoholism causes extensive damage to its victims and their families and results in significant costs for society through its associated morbidity and mortality. Early recognition and treatment are beneficial for the individual and cost-effective for society (1, 2). Sensitive, specific, rapid, and inexpensive methods for identifying individuals at risk of alcoholism in different populations are needed. Numerous procedures to detect heavy drinkers have been developed during the past 30 years. Conventional laboratory tests for γ-glutamyltransferase (GGT), mean corpuscular volume, aspartate and alanine aminotransferases, α-lipoproteins, and ferritin have been used for many years as biochemical markers of alcohol abuse (3) but have low diagnostic sensitivity and specificity. In 1976 Stibler and Kjellin (4) reported a qualitative change in isoforms of transferrin in cerebrospinal fluid and serum of patients with alcohol-related cerebellar tremor (4). Transferrin, the iron-transporting protein in blood, is a glycoprotein with two bi- or triantennary carbohydrate chains, each terminated with two or three sialic acids (SA; N-acetylneuraminic acid), respectively (5). The isoforms related to alcohol abuse contain less SA than other isoforms and can therefore be distinguished according to charge (4).

Several techniques for separation of isoforms have been introduced but they are generally laborious, non-quantitative, or expensive: chromatofocusing (6); disposable minicolumns combined with RIA (7); and electrofocusing followed by immunofixation (8), Western blotting (9), or zone immunoelectrophoresis (10). Results from more than 20 laboratories have recently been summarized (11).

Isoelectric focusing separates normal transferrin with high resolution into isoforms, depending on iron saturation, SA content, or amino acid substitutions (12). After complete iron saturation (2 Fe atoms per molecule), transferrin normally is separated into four isoforms according to their approximate isoelectric point (pI): pI 5.2 (5 SA), pI 5.4 (4 SA, major fraction), pI 5.6 (3 SA), and pI 5.7 (2 SA). The pI 5.7 fraction is normally < 0.8% of total transferrin, but may be increased more than 10-fold after heavy alcohol consumption. After excessive drinking an additional pI 5.9 fraction (0 SA) may appear. The pI 5.7 and pI 5.9 fractions are called carbohydrate-deficient transferrin (CDT). The purpose of this investigation was to develop an HPLC method for routine laboratories to identify subjects at high risk for alcohol (ethanol) dependence and to evaluate its sensitivity and specificity for detecting heavy alcohol consumption in defined populations.

Materials and Methods

Subjects

Subjects included 11 teetotalers, 45 occasional drinkers, healthy laboratory technicians (75% women; GGT values not available), and 284 men (ages 50–60 years) found to have high serum GGT activity in a general population screening 10 years ago (13). We reanalyzed samples for GGT; 43% of the patients had abnormal values, but there was no correlation between CDT and GGT. Twenty percent of the patients declared an alcohol consumption of > 40–70 g/24 h. We also analyzed intoxicated and detoxified patients.

Alcohol-intoxicated patients. Sixty heavy drinkers (59 men, 1 woman), ages 29–74 years, were all alcohol-dependent according to the Diagnostic and Statistical Manual of Mental Disorders (14), and had all previously been repeatedly detoxified. Only one of the patients had a diagnosis of alcoholic liver cirrhosis. No patient had diabetes mellitus, anemia, or other serious illness. The drinking pattern consisted of periods of heavy drinking alternated with periods of total sobriety. The current drinking period ranged from 1 week to > 4 months and

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3. Nonstandard abbreviations: GGT, γ-glutamyltransferase; SA, sialic acid; pl, isoelectric point; CDT, carbohydrate-deficient transferrin; IEF, isoelectric focusing; and TFC, transferrin variant C.

Received February 2, 1993; accepted June 3, 1993.
consisted of an estimated average daily alcohol intake of 200 g of ethanol (approximate range: 70–500 g).

A blood sample was drawn from each patient within 15 h after admittance. Four patients had blood drawn on days 0, 2, 4, 8, and 13 after their most recent alcohol consumption.

Detoxified patients. This group included 50 male alcoholics, ages 26–70 years, for whom a diagnosis of alcohol dependence had been previously established. Criteria of exclusion were diabetes mellitus, liver cirrhosis, anemia, and other serious illness. These patients had been sober for at least 2 months (range: 2 months–1 year) during ongoing aversion therapy (disulfiram or calcium carbimide) administered by nurses at the outpatient clinic at 2-day intervals. Relapse did not occur during this period, but we cannot exclude moderate drinking during treatment with calcium carbimide tablets.

Preparation of Samples

Fresh serum or serum frozen at −20 °C for <6 months was saturated with iron by adding 25 μL of NaHCO3 (500 mmol/mL) and 18 μL of FeCl3 (10 mmol/L Fe) per milliliter of serum. After mixing and storage at 8 °C overnight, the lipoproteins were precipitated by adding 10 μL of dextran sulfate (100 g/L) and 50 μL of CaCl2 (1 mol/L) per milliliter of serum. This mixture was stored for 30–60 min at 8 °C and then centrifuged at 10 000 × g for 10 min. The supernate was diluted fivefold with water and transferred to an HPLC autoinjector.

Equipment

The HPLC system consisted of pump no. 2941 (Pharmacia Biotechnology, Uppsala, Sweden), a Jasco (Tokyo, Japan) 870 UV detector equipped with a 460-nm filter, and a 10-mm flow cell together with a tungsten lamp. The system contained an autoinjector, Waters (Milford, MA) WISP 715, with a cooling system for 96 samples. A Shimadzu (Tokyo, Japan) CR 5A integrator was used for calculating the peak areas according to the valley-valley mode.

Procedure

Ion-exchange chromatography. The transferrin isoforms were separated on a Mono Q™ HR 5/5 column (Pharmacia) by salt gradient elution; the process took 32 min, including regeneration. Starting buffer (A) was Bis-Tris, 20 mmol/L, pH 6.2. Buffer B was buffer A plus NaCl, 350 mmol/L, at the same pH. Solution C, NaCl, 1 mol/L, was used for regeneration. Before use, all solutions were degassed and filtered through a 0.45-μm (pore-size) filter. Samples (200 μL) were injected, the flow rate was maintained at 1 mL/min, providing the gradient profile shown in Table 1, and the monitor sensitivity range was 0.01 A full scale.

Once a week the entire system excluding the Mono Q column was flushed with a 40 mmol/L solution of sodium hypochlorite. After 10–50 patients’ samples, the Mono Q column was regenerated according to the manufacturer’s instructions: pepsin (1 g/L) in acetic acid (100 mmol/L) containing sodium chloride (500 mmol/L) was pumped through the column with a retrograde flow rate of 1 mL/min for 10 min. The column ends were sealed and the whole column was stored at 37 °C overnight.

After the enzymatic treatment, conventional regeneration with sodium chloride, sodium hydroxide, and acetic acid was performed. The autoinjector was used to deliver the different solutions. Regeneration was terminated by antegrade flushing with solution C for 10 min. If the backpressure still exceeded 4 mPa, the top filter assembly in the column was replaced. The absorbance was registered at 460 nm and peak areas were automatically integrated. The sum of areas for all transferrin isoforms was considered as 100% and areas for individual isoforms were reported as a percentage of the total amount of transferrin.

Confirmation of the method: isoelectric focusing (IEF) of transferrin isoforms. Albumin was removed from serum by pretreatment with Blue Sepharose. Disposable columns (polypropylene, 5 mL; Pierce, Rockford, IL) were packed with swollen Blue Sepharose CL-6B (Pharmacia) (0.2 g, dry weight). After rinsing according to the manufacturer’s instructions, the small column was equilibrated with glycine (100 mmol/L), pH 7.2. We mixed 100 μL of the iron-saturated, lipoprotein-free supernate with 2 μL of β-mercaptoethanol (100 mL/L), and after 1 h at room temperature applied it to the minicolumn. Serum proteins were eluted with glycine (100 mmol/L), pH 7.2. The first 300 μL of the eluate was discarded; 40 μL of the next 800 μL of eluate was used for IEF. Each column can be used several times after regeneration with urea (6 mol/L), followed by glycine (0.1 mol/L), pH 7.0.

IEF was performed essentially as described for α1-antitrypsin (15) except that the ampholyte mixture was equal parts of Pharmalyte 4–6.5 and Pharmalyte 5–6 (total, 1.9 mL per 30 mL of gel solution). The same procedure can be performed on a smaller scale with the Phast System (Pharmacia). Transferrin isoforms can be verified by immunofixation with cellulose acetate membranes impregnated with transferrin antibodies (15). All pathological results from HPLC (CDT >0.8%) have so far been confirmed by IEF.

Quantification of serum transferrin. The serum transferrin concentration was estimated by electroimmunoassay (16) with antisera from Dako (Glostrup, Denmark).

Table 1. Gradient Profile for Ion-Exchange Chromatography of CDT

<table>
<thead>
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<th>Time, min</th>
<th>A</th>
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<th>C</th>
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<tr>
<td>0</td>
<td>100</td>
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<td>0</td>
</tr>
<tr>
<td>3.0</td>
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Results
Development of the Method

Transferrin isoforms have pl's -5.2-5.9. Optimal separation of the most cathodal isoforms was obtained at pH 6.2. The addition of NaHCO₃ together with FeCl₃ gave an optimal and stable iron saturation (12). Precipitation of lipoproteins improves the separation of the pH 5.9 isoform, which can be hidden under heavy β-lipoproteins in some patients. Fasted samples cannot be used with ambulatory alcoholics. Figure 1 illustrates HPLC elution patterns from teetotalers, occasional drinkers, and heavy drinkers; note the pl 5.9 isoform in the third pattern. The absorbance of the Fe-transferrin complex at 460 nm is ~10% of the 280 nm absorbance but is highly specific for the transferrin fractions. The amount of CDT at pl 5.7 represents only 0.2-0.8% (mean ± 2 SD) of the total transferrin in teetotalers and moderate drinkers. This value was slightly dependent on the method of integration of the chromatography profile. Slightly higher values were found with baseline integration, but the valley-valley method was more reproducible and less dependent on matrix effects. The CV's are given in Table 2.

IEF of CDT

Removal of albumin eliminates the need for immunofixation to visualize the specific transferrin isoforms. Lane S in Figure 2 represents an untreated serum sample with the diffuse albumin shadow that may sometimes cover the pl 5.7 isoform. Lanes 0, 2, 4, 8, and 13 show the complete disappearance of the pl 5.9 isoform and the gradually decreasing pl 5.7 isoform from day 0 to day 13. The IEF procedure is used to verify genetic variants that appear as abnormal chromatography profiles. All patients with CDT >1% showed increased intensity of the pl 5.7 fraction on IEF. Several genetic variants appeared, mostly subtypes of transferrin C together with a few cases of heterozygotes BC (lower pl) and CD (higher pl).

Catabolic Rate of CDT

The disappearance rate of CDT was determined by measuring the pl 5.7 CDT of four heavily intoxicated

<table>
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<th>Table 2. Precision of the Assay</th>
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<td><strong>Within-day</strong> (n = 7)</td>
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<td>Mean % CDT</td>
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<tr>
<td>Teetotalers</td>
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<tr>
<td>Moderate drinkers</td>
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Fig. 1. Elution profiles after ion-exchange chromatography on a Mono Q column of serum from: A, a heavy drinker (300 g alcohol/24 h, CDT 14.5%); B, a moderate drinker (70 g alcohol/24 h, CDT 2.4%); and C, a teetotaler (CDT 0.8%) The shaded peaks show the carbohydrate-deficient isoforms pl 5.7 (*) and pl 5.9 (**). The absorbance of the Fe-transferrin complex was measured at 460 nm.

Fig. 2. Isoelectric focusing of normal serum without removal of albumin (S) or with albumin removed by pretreatment with Blue Sepharose (N, 0, 2, 4, 8, and 13) Lane N represents a normal individual; lanes 0-13, a highly intoxicated patient on aversive therapy (days 0, 2, 4, 8, and 13). The approximate pl of each isoform is given to the right. Anode at the top; gradient pH 4-8.5.
hospitalized patients sequentially for 15 days, during which time no relapse of alcohol abuse occurred. The total transferrin concentration was measured in each sample (g/L) and CDT was calculated in mg/L in a semilogarithmic diagram (Figure 3). Total transferrin concentration increased with time in some patients during hospitalization. The half-life was determined from the linear slope of each curve. There was little difference between individual patients; the mean half-life was 9.5 ± 1 days.

Clinical Evaluation

CDT values found in samples from teetotalers and occasional drinkers (laboratory staff) showed a normal distribution and were consistently <1% by valley–valley integration (Figure 4). Among the 284 men from the general city population with a previous record of high GGT values, 20% declared an alcohol consumption of 40–70 g/24 h. In those men the sensitivity of CDT was 55% and the specificity 91%, for the cutoff value of 0.8%. Among the heavily intoxicated drinkers (70–500 g/24 h) the sensitivity was nearly 100%. Normalization of CDT was seen in 84% during aversion therapy. Some of these patients have not yet reached their basal CDT level. We cannot exclude moderate drinking during treatment with calcium carbimide tablets.

Discussion

Analytical Method

This HPLC method with ion-exchange chromatography is similar to the chromatographic system used for the glyated hemoglobin A1c, which is used for monitoring diabetes treatment (17). The method gives reproducible results and can be automated for large sample series. Forty patients’ samples can be analyzed in 24 h. The costs for reagents, including investments for HPLC, are ~30% of the reagent costs for the disposable minicolumns combined with RIA (Pharmacia Diagnostics). The latter technique must be run in duplicate. Another advantage of HPLC is the visible document of the specific 460 nm absorbance, which is important in detecting the genetic variation of transferrin. Sera from alcoholics are often lipemic and some lipoproteins and other serum
proteins may precipitate at pH 6.2, causing a gradual increase in column pressure. It is therefore convenient to use two columns so analyses can be performed during regeneration of one column. This approach has provided us with > 1 year of experience (> 1000 samples) with no problems. Genetic variants of transferrin are estimated to be present in about 29% of the population (18). Most of them represent subtypes of the major transferrin variant C (TFC) phenotype with minor changes in pls. They do not interfere with the chromatography pattern. Only the most anodal forms of TfBC heterozygotes and TFCD heterozygotes, frequency 1–2 per 1000 in the Caucasian population (19), will interfere with the chromatography profile. In these cases it is necessary to confirm the results with IEF in a specialized laboratory.

The advantage of the transferrin index has been recently discussed (20). Our procedure automatically gives the calculated percentage of CDT from the integrator and eliminates the need for specific determination of transferrin concentration. The observation of the large variation of total transferrin concentration in women with increases due to iron deficiency and estrogen administration and in highly intoxicated alcoholics (range 1.1–3.6 g/L) suggests that the CDT percentage of total transferrin rather than absolute quantities be used. The half-life of ~9.5 days for the pl 5.7 isoform allows evaluation of alcohol consumption during the past 1–3 weeks or verifies a successful treatment. This result is in better agreement with the published half-life of 8–10 days for normal transferrin (21) than the estimated 15 days for CDT (6).

Structural studies of the carbohydrate chains of CDT isoforms remain contradictory. There are three possibilities: missing terminal sialic acids (22), missing trisaccharide chains (23), or missing whole carbohydrate chains, the last of which was recently shown for the carbohydrate-deficient glycoprotein syndrome (24). However, the final effect at ion-exchange chromatography or IEF will be the same.

Sera from patients with carbohydrate-deficient glycoprotein syndrome (25) show a similar and a more pronounced increase of isoforms pl 5.7 and 5.9. The fraction of transferrin in cerebrospinal fluid has the same position, both in chromatography and IEF, as our present pl 5.9 isoform. All glycoproteins with carbohydrate chains terminating with sialic acid are very sensitive to contamination with the enzyme neuraminidase, which is produced by some bacteria and viruses. The addition of sodium azide, final concentration 2 g/L, to all serum samples before transport or storage for longer times at room temperature is therefore recommended. When the CDT concentration is increased, it is a very specific marker for alcohol abuse and greatly superior to other currently available biological markers. False-positive results (slight to moderate CDT increases) have been seen in 8% of the patients with primary biliary cirrhosis or heterozygotes for TFCD (26). More experience is necessary to evaluate CDT in different clinical conditions such as cirrhosis and malignancy.

Clinical Application

Depending on the examined population, the sensitivity and specificity of CDT varies. According to Stibler (11), a sensitivity of 82% and a specificity of 97% have been achieved with all the methods used so far. In a population examined at a general medical practice (27) in which the subjects had a wide range of alcohol consumption, the sensitivity decreased to ~45%. This has also been the case in two reports from the general population in Norway (28) and Finland (29). Quite a low sensitivity, especially in young individuals, was found in the Finnish study. In our evaluated population of middle-aged men, the individuals have been very thoroughly characterized and followed over 15 years. At the follow-up in 1991, the alcohol consumption was assessed by two trained nurses and blood was sampled for CDT at the same occasion. We found a sensitivity of 55% in the group that reported an alcohol consumption of > 40 g/day. However, in the study of intoxicated individuals consuming > 70 g/day, the sensitivity was near 100%, which is in agreement with Stibler’s results (11). The high specificity in the teetotalers and the occasional drinkers, near 100%, allows the conclusion that CDT has the highest specificity of the available biological markers.

The skillful assistance of Anna Arnetorp, Benny Larsson, and Kerstin Ekström is gratefully acknowledged.

References