Quantification of carbohydrate-deficient transferrin by ion-exchange chromatography with an enzymatically prepared calibrator

Florian Renner1* and Rolf-Dieter Kanitz2

The current HPLC method for the determination of carbohydrate-deficient transferrin (CDT) yields ratios of CDT isoforms in relation to total transferrin, whereas the use of absolute concentrations obtainable in routine analysis by RIA and the reference ranges based hereupon is more convenient. We describe a modified HPLC method that likewise gives absolute CDT concentrations by using a calibrator prepared by treatment of transferrin with neuraminidase. Separation of isoforms could be improved and analysis time reduced to ~2 h. Iron saturation proved stable during chromatography. In contrast to a commercial RIA, the cheaper and more time-saving HPLC method excludes erroneous results caused by aged samples or genetic transferrin variants and enables the determination of asialo- and disialotransferrin. Both methods showed comparable precision and correlated with each other (y = 1.76 + 0.27x; Sdp = 5.38); for the HPLC method precision was 1.3–9.8% (within assay) and 6.2–10.6% (between assay). The clinical evaluation with a cutoff concentration of 80 mg/L resulted in a diagnostic specificity of 100% and a sensitivity of 82.5%.

INDEXING TERMS: desialylated transferrin • neuraminidase • alcohol abuse marker • high-performance liquid chromatography • radioimmunoassay • isoelectric focusing

Carbohydrate-deficient transferrin (CDT) is considered a reliable marker of chronic alcohol abuse [1–3]. However, CDT is not a single molecular entity but refers to a collective of sialic acid-deficient transferrin isoforms, which are mainly di- and asialotransferrin [4]. Whereas commercially available RIAs still requiring cumbersome sample pretreatment do not discern single CDT isoforms, isoelectric focusing (IEF) combined with Western blotting is regarded as a reference method [5, 6]. However, its sophisticated technique precludes this method from routine use.

Alternatively, a HPLC method for routine laboratories based on ion-exchange chromatography was developed by Jeppsson et al. [7], yielding ratios of CDT isoforms in relation to total transferrin (relative CDT values). Nevertheless the use of absolute concentrations for the CDT isoforms (absolute CDT values) obtainable after calibration and the reference ranges based hereupon is more convenient.

Hence, we describe a modified HPLC procedure, yielding absolute results, based on quantification with desialylated transferrin prepared from immunologically pure transferrin (IPT) by neuraminidase treatment. Furthermore, we compared this method with the most used RIA (CDTect™).

Materials and Methods

PATIENT SELECTION
We investigated 40 currently drinking alcohol-dependent inpatients (33 males, 7 females), all admitted for detoxification in the psychiatric clinic. As comparison groups we selected 34 sober alcohol-dependent inpatients (22 males, 12 females) with secured abstinence of >2 weeks and 39 psychiatric inpatients without any alcohol consumption (teetotalers). The blood samples in the currently drinking group were obtained at the day of admission. All investigated patients gave their informed consent to the study.

APPARATUS
Photometric measurements were performed with a digital photometer 6114 S (Eppendorf Netheler Hinz, Hamburg, Germany). pH was adjusted with a precision pH-mV meter pH 531 (WTW, Weilheim, Germany) and a combination pH electrode Ingold 405-S7/120 (Mettler–Toledo, 1 Institut für Klinische Chemie and 2 Klinik für Psychiatrie, Medizinische Universität zu Lübeck, Ratzeburger Allee 160, 23538 Lübeck, Germany *Author for correspondence. Fax 0451/5002904.

3 Nonstandard abbreviations: CDT, carbohydrate-deficient transferrin; IEF, isoelectric focusing; IPT, immunologically pure transferrin; Bis-Tris, 2-bis(2-hydroxyethyl)amino-2-(hydroxymethyl)-1,3-propanediol; MIT, 2-methyl isothiazolone; and PAGE, polyacrylamide gel electrophoresis.

Received May 6, 1996; revised October 2, 1996; accepted October 30, 1996.
Urdorf, Switzerland). A Thermo Mixer 5433 (Eppendorf Netheler Hinz) with a mixing frequency of 1400 min⁻¹ was used for incubation. The chromatographic system consisted of an AS-2000 A autosampler with a 200-µL sample loop, a L-6220 intelligent pump, a L-5025 column heater, a L-4250 UV-VIS detector, and in some cases a L-4500 A diode array detector (all from Merck, Darmstadt, Germany). All solutions used for chromatography were degassed with an ERC-3315 degasser (ERC, Alteglofshaim, Germany). System controlling and calculations were done with the D-7000 HPLC Manager software (Merck). Anion-exchange chromatography was performed with a Mono-Q-HR 5/5 column (Kabi Pharmacia Diagnostics, Uppsala, Sweden) connected to a prefilter for HR 5/5.

REAGENTS
2-Bis(2-hydroxyethyl)amino-2-(hydroxymethyl)-1,3-propanediol (Bis-Tris) was purchased from Aldrich (Steinheim, Germany), 2-methylisothiazolone (MIT) from Boehringer Mannheim (Mannheim, Germany), ferric citrate monohydrate from Fluka (Buchs, Switzerland), and dextran sulfate from Immuno (Heidelberg, Germany). IPT was kindly supplied by Behring (Marburg, Germany).

The purity of IPT was confirmed in several tests (P. Merle, Behring): By using either anti-human antiserum (rabbit) or antiserum against transferrin (rabbit), only one precipitation line was obtained by immunoelectrophoresis; microzone electrophoresis and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) demonstrated purity >98%, and with PAGE the position of the tetrasialotransferrin isoform was identical to the one of a fresh control serum; the iron-binding capacity of 12.5 µg of Fe/10 mg of IPT as confirmed by IEF agreed with the calculated amount. Insoluble neuraminidase type II-A from Vibrio cholerae and type VI-A from Clostridium perfringens were from Sigma (Deisenhofen, Germany). All other chemicals were purchased from Merck. Water of highest purity was obtained from a Milli-Q UF unit (Millipore, Eschborn, Germany). Eluents were prepared as follows. A: Bis-Tris, 20 mmol/L; MIT, 0.66 mmol/L; B: Bis-Tris, 20 mmol/L; MIT, 0.66 mmol/L; sodium chloride, 300 mmol/L; C: sodium chloride, 1 mol/L. pH of A and B were adjusted with 20% acetic acid to 6.52 ± 0.01. All eluents were filtered through a 0.45-µm (average pore size) filter and degassed before use. The bacterostatic reagent MIT was added to eluents with nitrogen-containing buffers to prevent bacterial contamination. However, measurements at 280 nm detection wavelength were realized with only fresh-prepared eluents without MIT to avoid eluent absorption. The buffer for reactions with neuraminidase consisted of 100 mmol/L sodium acetate and 20 mmol/L calcium chloride at pH 6. Protein concentrations were measured with Biuret reagent according to Weichselbaum [8] in a 20-mm cuvette with use of a 6 g/L calibrator.

SAMPLE PREPARATION
Serum samples had been kept at most 4 h at room temperature before storing at −28 °C. Sample preparation largely followed the method of Jeppsson et al. [7] with minor modifications. These were use of more-concentrated reagents for iron saturation, a reduced saturation time, and changed volumes for better practicability. Serum (100 µL) was saturated with iron by adding 3 µL of 500 mmol/L sodium hydrogen carbonate and 2 µL of 50 mmol/L ferric citrate, followed by precipitation of β-lipoproteins after 30 min and dilution.

PREPARATION OF CALIBRATOR
IPT (40 mg) was incubated with 100 mU of insoluble neuraminidase type II-A from V. cholerae, 200 mU of insoluble neuraminidase type VI-A from C. perfringens, and 5 mL of buffer under constant stirring at 37 °C for 72 h. The insoluble enzymes were removed by centrifugation. A protein concentration of 7.84 g/L before and 7.88 g/L after treatment with neuraminidase was determined. The stock solution was stored at −78 °C.

CHROMATOGRAPHY
Chromatography was performed at 20 °C essentially according to Jeppsson et al. [7], but using eluents with a slightly different pH and a changed gradient profile (Table 1). Integration was performed by the valley-to-valley method except for disialo- and trisialotransferrin peaks (see Fig. 1A and B). After 200 samples the column was regenerated according to manufacturer’s instructions; cleaning with pepsin was only performed if the column performance was still not restored after the regeneration.

DETERMINATION OF ABSOLUTE CDT CONCENTRATIONS
The stock solution of the calibrator (see above) was diluted 1:16 with water and saturated with iron. For quantification of CDT by HPLC, different volumes of this mixture were injected.

RIA
CDTect (Kabi Pharmacia Diagnostics) was performed according to the instructions of the manufacturer. After

| Table 1. Gradient profile used to separate transferrin isoforms. |
|---|---|---|---|
| Time, min | Eluent A, % | Eluent B, % | Eluent C, % |
| 0.0 | 90 | 10 | 0 |
| 3.0 | 90 | 10 | 0 |
| 3.1 | 85 | 15 | 0 |
| 7.0 | 85 | 15 | 0 |
| 18.0 | 70 | 30 | 0 |
| 18.1 | 0 | 0 | 100 |
| 22.0 | 0 | 0 | 100 |
| 22.1 | 90 | 10 | 0 |
| 32.0 | 90 | 10 | 0 |
iron saturation of the serum samples, isolation of transferrin isoforms with pIs ≥ 5.7 (asialo-, monosialo-, and disialotransferrin) is accomplished by anion-exchange chromatography on microcolumns. Quantification is done by double-antibody RIA with results given in arbitrary units (U/L).

**STATISTICAL ANALYSIS**

Regression analysis was done according to Passing and Bablok [9]. Clinical results were analyzed by using the Statistical Package for Social Sciences (SPSS, Chicago, IL).

**Results**

Figure 1 shows chromatograms obtained with the modified HPLC method, yielding ratios of transferrin isoforms in relation to total transferrin. Whereas isoforms with 2, 3, 4, and 5 sialic acid residues can be detected in samples of healthy controls (1A), in cases of severe alcohol abuse an additional signal belonging to asialotransferrin appears in the chromatogram (1B). As is demonstrated in Fig. 1C, samples stored for extended time at room temperature show widened peaks at atypical positions, indicating in vitro degradation of carbohydrate chains. Absolute CDT concentrations were determined by using a calibrator prepared by treatment of IPT with neuraminidase (Fig. 1D). The preparation procedure was optimized by minimizing enzyme concentration and increasing incubation time to give high yields. The results of quantification with four different concentrations of the calibrator are shown in Fig. 2. A factor of 3.8 was calculated for the conversion of U/L (measured by RIA) to mg/L (measured by HPLC), comparing replicate measurements (n = 10) of the calibration preparation and a patient sample.

Precision of the RIA is shown in Table 2, referring to data given by the manufacturer of the kit and confirmed in other studies [10, 11]. By examining precision of the HPLC method with pooled sera with CDT concentrations...
close to those used for the evaluation of the RIA, comparable CVs were obtained. Low within-assay values of <2% were achieved at high CDT concentrations. Precision and sensitivity of the HPLC method were best with baseline integration for disialo- and trisialotransferrin peaks and otherwise the valley-to-valley method. The detector response was linear up to the highest concentration tested of 3000 mg/L. By using diluted serum samples, a detection limit (signal-to-noise ratio of 3) of 10 mg/L was determined. To examine the correlation between the HPLC method and the RIA, 113 patient samples were analyzed with both methods (Fig. 3). Regression analysis showed correlation of the methods with each other. With the linear regression line a conversion factor of 3.7 is obtained, close to the value of 3.8 mentioned above. One sample yielded markedly different values exhibiting an atypically high peak between asialo- and disialotransferrin; results from this sample were excluded from statistical analysis shown in Fig. 3.

For the accuracy of the HPLC method, stability of iron saturation during the whole chromatographic process is required [12]. This could be proved by diode array detection and comparison of the absorbance at 460 nm (for transferrin) and at 280 nm (for all proteins). With IPT as sample (n = 8), a quotient (absorbance_{460nm}: absorbance_{280nm}) of 0.045 was obtained for all isoforms.

Clinical evaluation of the HPLC method was performed, investigating three groups of subjects with different alcohol consumption (Fig. 4). Referring to results of Godsell et al. [13], we used 80 mg/L as cutoff concentration. In the highly selected groups the diagnostic specificity for the absolute CDT values was 100%, whereas the diagnostic sensitivity reached 82.5%.

**Discussion**

The HPLC method developed by Jeppsson et al. [7] is based on ion-exchange chromatography. Transferrin isoforms with different contents of sialic acid residues can be distinguished according to their charge [5]. Newer findings indicate that the CDT isoforms also lack one or both of their entire carbohydrate chains [4]. The absence of any carbohydrate chain could explain the early chromatographic elution of asialotransferrin, leading to an excellent separation from the other isoforms.

A disadvantage of this method is that only ratios of CDT isoforms in relation to total transferrin are measured. According to Stibler et al. [1, 3], such ratios may give rise to both false-positive and false-negative results, owing to independent variations in total transferrin concentration since total transferrin has been found to be unrelated to CDT. Therefore we described a modified HPLC method that allows the determination of absolute concentrations also. For the purpose of quantification we used a calibrator prepared by treatment of IPT with neuraminidase. Between-assay precision was slightly better as reported from Jeppsson et al. [7], which could be a result of realized modifications. Changes in gradient profile and pH resulted in superior separations of isoforms with the difference in retention time improved for disialo- and trisialotransferrin from ~1 min to 2 min. Integration of chromatograms by using a combination of baseline and valley-to-valley methods gave best precision and sensitivity. Cutting time for iron saturation from 16 h to 30 min allows first results after 2 h.

Stability of iron saturation could be proved when
comparing absorption at detection wavelength 460 nm and 280 nm. A quotient (absorbance_{460nm}/absorbance_{280nm}) of 0.045 was obtained for all isoforms, being in agreement with results of Storey et al., who determined 0.046 [14, 15]. The absorbance at 280 nm arises from the aromatic amino acids and should not be influenced by changes in the carbohydrate structure. The finding that the quotient is the same for all isoforms is consistent with the hypothesis that the absorbance at 460 nm is also not influenced by changes in the carbohydrate structure.

Compared with a commercial RIA, the cheaper and more time-saving HPLC method provides comparable results. The detection limit of 10 mg/L (12.5% of cutoff concentration) is higher, as for the RIA with 1 U/L (5% of cutoff concentration). However, it should be sufficient for the detection of alcohol abuse and is comparable with results of Schellenberg et al. [16]. Since appropriate anion-exchange columns are available, the use of microbore HPLC should be preferred for the realization of lower detection limits—desirable, for example, for the detection of asialotransferrin in nasal fluid if cerebrospinal fluid leakage is suspected [17, 18].

In contrast to the RIA, the identification of aged samples (see Fig. 1C) and genetic transferrin variants, both leading to erroneous CDT results, is possible [7]. Transferrin indices (disialotransferrin:tetrasialotransferrin) can be calculated without further measurement of total transferrin [19].

The clinical evaluation with a cutoff concentration of 80 mg/L [13] resulted in a diagnostic specificity of 100% and a sensitivity of 82.5% when examining a group of alcohol-dependent inpatients who were admitted for detoxification. These findings are in good agreement with earlier results. Stibler [1] reported a specificity of 97% and a sensitivity of 65–95%. Godsell et al. [13] detected 86% of an alcohol-dependent group with CDT values >80 mg/L on the basis of IEF measurements and calculation of absolute CDT values via percentage of total transferrin, estimated by immunonephelometry. Schellenberg et al. obtained a sensitivity of 73% and a specificity of 90% with 70 mg/L as cutoff concentration on the basis of nephelometric measurements [16]. With the use of relative CDT values measured by HPLC and 0.8% as upper reference value, a sensitivity of nearly 100% among heavily intoxicated drinkers and 54% among patients with a declared alcohol consumption of 40–70 g/24 h is reported by Jeppsson et al. [17]. However, all these findings depend on the examined population, and authors referring to community screening reported lower sensitivities [20]. Therefore we are presently examining an unselected group of surgery patients for establishing valid reference ranges.

So far only CDT concentrations are used for the detection of alcohol abuse. The quantitative measurement of asialotransferrin available by HPLC could lead to increased specificity, which is important especially for forensic questions. The knowledge of the amount of each transferrin fraction would be helpful for the diagnosis of carbohydrate-deficient glycoprotein syndrome and disialotransferrin development deficiency syndrome [21, 22].

Up to now it was only possible to measure relative and absolute CDT values when using two different RIA kits or IEF. The HPLC method measures both with appropriate expenditure. This could be helpful to avoid the increased rate of false-positive CDT results in certain clinical collectives with increased total transferrin as reported especially for females in late pregnancy [23].

The additional use of relative CDT values may be appropriate for screening for hazardous consumption among specific populations with marked alteration in the total transferrin [13]. Whether a combination of relative and absolute results will increase clinical sensitivity and specificity of the parameter remains to be proved [16, 24].

Our thanks are due to K. Stratmann, B. Gütschow, and D. Schult for their skillful assistance and to L. Dibbelt for methodological discussions. Immunologically pure transferrin was generously supplied by S. Baudner (Behring, Marburg, Germany).

References

4. Landberg E, Pålsson P, Lundblad A, Arnetorp A, Jeppsson JO. Carbohydrate composition of serum transferrin isoforms from...


