We describe a technique for measuring carbohydrate-deficient transferrin (CDT) in serum. Serum transferrin fractions are separated by anion-exchange chromatography on microcolumns. Sialic acid-deficient transferrin fractions are collected in the eluate, and transferrin is then quantified by a rate-nephelometric technique. Imprecision (CV) was 4–5% within-run and 7–9% between runs (n = 15). Comparison with an isoelectric focusing-immunofixation method for transferrin index (x) yielded y = 761x + 7. S_x = 39 mg/L. Assay of sera from 90 abstainers or moderate consumers of alcohol showed that 81 (90%) had CDT concentrations between 30 and 70 mg/L. Among 74 alcoholics admitted to an alcohol treatment center, 54 (73%) had CDT >70 mg/L, i.e., the diagnostic sensitivity was 73% at a specificity of 90% (area under receiver-operator characteristic curve = 0.891).

INDEXING TERMS: abused drugs • alcoholism • rate nephelometry • toxicology

An increase in serum transferrin fractions with an isoelectric point (pI) ≥5.7 has been observed in cerebrospinal fluid and serum of alcoholic patients [1]. Other studies have demonstrated that this anomaly is caused by differences in the composition of the carbohydrate part of the protein, such that both the sialic acid [2] and the galactose [3] contents are decreased. Measurement of carbohydrate-deficient transferrin (CDT) has been proposed as a marker of alcohol abuse [4–18] and of abstinence [2, 10, 19, 20]. Among the methods developed for determining CDT, isoelectric focusing remains the technique of choice [4, 5, 7, 21–26] but is generally not suited for routine use [27]. A newly described semiautomated method [28, 29] based on iso-electric focusing seems much more suitable for routine use. Chromatofocusing [30] and HPLC [31] have also been proposed. Anion-exchange chromatography followed by RIA or enzyme immunoassay is potentially useful [6, 9–15, 17–20, 32, 33] but is more suitable for analyzing large series than for single determinations or small series. We have developed a reliable and rapid method for assaying CDT, based on rate-nephelometric determination after anion-exchange separation.

MATERIALS AND METHODS

NEPHELOMETRIC METHOD

Principle. After serum transferrin is iron-saturated by incubation with a solution of ammonium iron sulfate, the proteins are separated according to their pI values by anion-exchange chromatography on disposable columns. Because fractions of transferrin with low sialic acid content have a higher pI than normally sialylated transferrin, the low-sialic fractions are eluted first. Transferrin in the eluate is then quantified by rate nephelometry.

Reagents. All reagents were of analytical grade. Piperazine hexahydrate (cat. no. 7327), formic acid (cat. no. 263), and ammonium iron sulfate hexahydrate (cat. no. 3862) were supplied by E. Merck (Darmstadt, Germany). Neuraminidase (EC 3.2.1.18; cat. no. ORKD) was provided by Behringwerke (Marburg, Germany), Polybuffer exchanger 94 (PBE 94; cat. no. 17-0712-01) by Pharmacia Biotech (Uppsala, Sweden), and polyethylene glycol (PEG; M, 6000, 400 g/L, cat. no. 4440011) by Immuno (Heidelberg, Germany). Human transferrin (cat. no. 449560), polyclonal goat antiserum directed against human transferrin (cat. no. 449420), and liquid control VIGIL PRx Level 2 (cat. no. 450125) were obtained from Beckman Instruments (Brea, CA).

We tested three different lots of antiserum in this study; all gave similar calibration curves and had a protein concentration <6 g/L.

ANALYTICAL PROTOCOL. One buffer was used for dilution of the sample, conditioning of columns, and elution (piperazine, 20 mmol/L, adjusted to pH 5.7 with formic acid). After bringing
PBE 94 gel to room temperature over at least 1 h, we homogenized the suspension with slow mixing. We then washed the gel by adding 10 volumes of the buffer and gently mixing. After sedimentation, preservative agents and damaged particles were eliminated by aspirating the supernate. We repeated this step, then added to the gel an equal volume of buffer and used this to fill 0.8-mm-diameter disposable polystyrene microcolumns (cat. no. 29920; Pierce Chemical Co., Rockford, IL) with ~3 mL of the slurry so that the height of the gel in the column was 2.7 cm. After all the liquid had passed through the column, we placed a polyethylene disk on the surface of the gel and applied pressure to compress the gel height in the column to 2.4 cm. The columns were then ready for use.

The filled columns can be stored for 10 days at 4 °C. Sera can be kept at 4 °C for 2 days or at ~20 °C for 6 months.

The sera were iron-saturated by incubating 100 μL of serum with 300 μL of ammonium iron sulfate (6 g/L) for 90 min at room temperature; after this, 600 μL of buffer was added to yield a final 10-fold sample dilution. We applied to each column 1 mL of pretreated sample and, after all of the sample had been adsorbed, poured 0.5 mL of buffer on the column. The eluate was discarded (void volume). We then poured 1.5 mL of buffer on the column and collected the eluate, which contained proteins with pI >5.65. The pI of the eluted proteins was monitored by isoelectric focusing in parallel with reference pI calibrators.

Nephelometric measurements were performed with an Array 360 Protein System (Beckman Instruments): 400 μL of eluate was incubated with 100 μL of 400 g/L PEG for 10 min, centrifuged for 5 min at 4000g, placed in the external well of the dilution segments, and quantified for transferrin by a user-prepared chemistry (gain = 33, dilution = “near 100 μL”) with use of a 100-μL sample volume, 500 μL of buffer, and 42 μL of undiluted antibody. While monitoring the antigen–antibody reaction, the system determines the first derivative of scattered light produced. The rate signal reaches a maximum at the point at which the peak rate-of-change in the scatter signal occurs. Thus, the initial scatter contributed by the sample-plus-antibody background does not affect the rate measurement. Results are obtained after ~50 s of reaction and are expressed in rate signal units (RT).

The calibration curve was plotted from measurements of a commercial calibrator (Beckman Instruments cat. no. 449560; transferrin concentration 5.95 g/L) serially diluted with buffer to give final transferrin concentrations of 2–10 mg/L. Calibrators underwent the same PEG pretreatment as eluates.

ISOELECTRIC FOCUSING

Isoelectric focusing was undertaken as previously described [7, 23]. We prepared 0.5-mm-thick gels without ampholytes, dried them completely, stored at room temperature, and soaked them in the mix of ampholytes before use. After overnight soaking, they were partially dehydrated to eliminate excess humidity. Sera were iron-saturated [34] and their transferrin concentrations were adjusted to 40 mg/L. After focusing and direct immunofixation [21], the transferrin–antibody complex was stained and quantified by densitometry. Results were expressed as the ratio between disialotransferrin (pI 5.7) and tetrasialotransferrin (pI 5.4), referred to as the Tf index [7].

POPULATIONS STUDIED

All procedures were accepted by the ethical committees of the Regional Institute for Health and of the Centre Louis Sevestre.

Controls. Sera were collected from 90 apparently healthy males, ages 14–59 years (mean 44 years), who had undergone medical examinations at the Regional Institute for Health. Their mean weekly alcohol consumption ranged from 0 to 150 g. Blood was collected the morning after overnight fasting. Subjects included in this study considered themselves to be healthy, and all worked full time. They received a regular and unrestricted diet, their body mass index was normal, and they were not hyperlipemic.

Alcoholics. The sera of 74 male alcoholic patients admitted to an alcohol treatment center (Centre Louis Sevestre) were assayed. Their ages ranged from 21 to 66 years (mean 47 years). Blood was collected the morning after admission. They had not voluntarily reduced their alcohol consumption before their admission and did not present clinical or biological signs of liver disease. Their mean daily alcohol consumption during the month preceding admission was 80–250 g.

ASSAY EVALUATION

Detection limit. The detection limit of the technique was evaluated by measuring 10 blank samples, i.e., 1:4 (by vol) mixtures of 400 g/L PEG and elution buffer, as sample and calculating the mean ± 3 SD of these measurements.

Precision. Within-run imprecision was evaluated by 15 repeated measurements of three serum pools with different CDT concentrations in the same series. Between-run imprecision was evaluated by repeated duplicate measurements of the same three pools on 15 nonconsecutive days. The total variation was calculated from all of the results for each pool. The within-assay imprecision of the isoelectric focusing–immunofixation method was evaluated by making 10 measurements on two different gels; the between-run imprecision was the result of 10 duplicate determinations on 10 nonconsecutive days.

Linearity and recovery. The linearity of the technique was assessed with the use of a totally desialylated serum, prepared by incubating 100 μL of serum for 24 h at 37 °C with an equal volume of neuraminidase reagent (100 U/L) [2]. After saturating the desialylated serum with iron [34], we diluted it severalfold (2:100 to 40:100 by vol) with a normal serum and analyzed the dilutions in triplicate. The recovery of the chromatographic step was estimated by nephelometric measurement of transferrin in a desialylated serum before and after passage through the anion-exchange column.

Results

When submitted to isoelectric focusing in parallel with serum samples, the eluates appeared to contain almost all of the asialo-
and monosialotransferrin, the major part of the disialotransferrin, and a slight contamination with trisialotransferrin.

Figure 1 shows a calibration curve obtained with serial dilutions of the transferrin calibrator. The use of logarithmic scales for plotting the CDT concentration in the eluate after addition of PEG (x) and the nephelometric measurement (y; in RT) allows better appreciation of low measurements. The calibration curve is quasilinear from 2 to 10 mg/L (linear regression analysis: log y = 1.449 log x - 0.274, r² = 0.998). The stability of the calibration curve was tested by repeated determinations of the same sera for 2 months. No significant shift was observed during this period; thus, a new calibration is not required for each run. The initial dilution of sera was 10-fold, and 1 mL of sample volume corresponded to 1.5 mL of eluate, making the final dilution 1:15. Therefore, this method can measure CDT concentrations in serum of 30–150 mg/L. When a result exceeds this limit, one can either dilute the serum in the elution buffer or use 42 μL of sample volume (fixed volume of the Array Protein System) instead of 100 μL.

The method appeared linear (Fig. 2) for CDT concentrations ranging from 25 to 150 mg/L, i.e., throughout the range of measurement (y = 0.97x + 5, r² = 0.988).

For 10 determinations performed with elution buffer instead of serum, the apparent CDT values ranged from 0.8 to 5.4 mg/L (mean = 3.6, SD = 3.9). The mean + 3 SD of these measurements corresponded to 9.9 mg/L, which is less than the lowest point of the calibration curve (100 RT for 30 mg/L). We therefore estimate the detection limit to be <10 mg/L. Abnormally low values (CDT <20 mg/L), caused by abnormal pauses in the rate of the reaction, were sometimes seen. Repeat determinations and isoelectric focusing indicated that this occurred only in subjects with a low CDT concentration.

The imprecision (CV) of the nephelometric step was <4% for CDT values <60 mg/L and <2% for higher values.

Within-run imprecision of the assay varied from 4.9% for a CDT value within the normal range (30–70 mg/L) of the assay to 4.3% for a value near the upper limit of the technique. The between-run imprecision of the same pools ranged from 8.6% to 6.6% (Table 1), and total variations were respectively 7.8% and 6.3%.

Analysis of 50 samples by both anion-exchange chromatography–nephelometry and isoelectric focusing–immunofixation [7] (Fig. 3) yielded the regression equation CDT = 761 × Tf index + 7 (Sdix = 39 mg/L, Spearman’s rank correlation coefficient = 0.834).

Figure 4 indicates the distribution of CDT in 90 healthy male subjects—abstainers or moderate drinkers (Table 2). Most values were in the range 30–50 mg/L and only one was >100 mg/L: the mean CDT was 46 mg/L (SD 16.8). CDT concentrations were significantly higher in the alcoholic patients (Table 2): The distribution of their CDT values was broad (Fig. 4), from 50 to 120 mg/L, and some very high concentrations were present (>300 mg/L). Plotting the receiver-operating

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**Table 1. Within-run and between-run imprecision of chromatographic–nephelometric CDT assay and Tf index calculation at various CDT concentrations.**

<table>
<thead>
<tr>
<th>Sample</th>
<th>CDT, mg/L</th>
<th>SD</th>
<th>CV, %</th>
<th>Between-run</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SD</td>
<td>CV, %</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>SD</td>
<td>CV, %</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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* n = 15 for CDT determinations; n = 10 for Tf index.
* n = 15 (duplicate measurements) for CDT; n = 10 for Tf index.
* n = 45 (15 single measurements on the same day and 15 duplicate measurements on 15 nonconsecutive days).
characteristic curve from the CDT values of both groups (Fig. 4, inset) showed that, for an upper limit of normal value of 70 mg/L, the assay sensitivity was 0.73 and its specificity was 0.90.

Discussion

ANALYTICAL METHOD

A variety of analytical methods have been used to quantify CDT. Because no specific antibody against CDT has been produced to date, all methods involve a separation of the different transferrin isoforms and quantification of the CDT forms. CDT can be considered as the totality of the fractions with a reduced sialic acid content, i.e., di-, mono-, and asialotransferrin. None of the currently described methods takes into account all these fractions, even though the magnitude of the alcohol-induced increase of di-, mono-, and asialotransferrin is not the same. Consequently, analytical precision and diagnostic efficiency of the different methods can be compared, but not the numeric results for absolute or relative CDT concentrations.

Isoelectric focusing followed by immunofixation was the earliest method developed to quantify CDT [2, 4, 5, 7, 35–39]. The resolution of the bands produced is enhanced by using Western blot instead of immunofixation [24–26, 40, 41]. These methods are not yet adapted to a large number of determinations, and their interassay precision is not always satisfactory. Recently, Lof et al. [28, 29] proposed a semiautomated isoelectric focusing–immunofixation method that avoids these disadvantages. “FPLC” (Pharmacia) [29, 42] and HPLC [31] may also be used to separate the transferrin isoforms, and after separation, transferrin fractions can be quantified either by reading the absorbance directly (when the samples have been previously purified) or by using RIA to determine the transferrin in the collected fractions. All of these methods allow discrimination between an increase of CDT and the existence of a genetic C-D variant [43, 44] that contains transferrin fractions with a higher pI.

To simplify CDT measurement, Stibler et al. [6] developed micro anion-exchange chromatography to separate the transferrin components and quantified them by RIA. This method was later modified [45] by changing the elution buffer to enhance the interassay precision. Other variants of this method have been described, in which RIA [33] or enzyme immunoassay

Table 2. Concentrations of serum CDT in alcoholic patients and reference subjects.

<table>
<thead>
<tr>
<th>Reference subjects</th>
<th>Alcoholic patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>74</td>
</tr>
<tr>
<td>Age, years</td>
<td>44</td>
</tr>
<tr>
<td>CDT, mg/L</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>46</td>
</tr>
<tr>
<td>SD</td>
<td>16.8</td>
</tr>
<tr>
<td>Range</td>
<td>29–129</td>
</tr>
</tbody>
</table>

*Significantly different from reference subjects: P < 0.001.

Fig. 3. Correlation between CDT values and Tf index values in 50 individuals (alcoholic patients — and control subjects —).

Fig. 4. Distribution of CDT values in 90 reference subjects (white bars) and 74 alcoholic patients (shaded bars). Inset: Receiver-operating characteristic curve for CDT calculated from the values for these subjects and patients.
[46] is used to quantify transferrin. Ion-exchange chromatography on microcolumns elutes a variable quantity of each fraction, depending on the elution conditions (phase, buffer, sample volume). As a result, different methods, even when measuring CDT in comparable population samples, give different values.

From one study to another, the imprecision of the methods varies from 7% to 18% [6, 24, 28, 45, 46]. In the present method, within- and between-assay variations were strongly affected by the flow rate through the columns. This is why we defined the precise gel heights before and after compression. The pH of the buffer is another key point of the between-assay imprecision. The tolerance is only 5.7 ± 0.015 in the method we describe here.

**Comparison with Tf index**

Although CDT values correlated well with Tf index values, the scatter plot (Fig. 3) shows the existence of individual discrepancies, as has been seen in similar studies comparing different methods. This has to be interpreted by taking into account that the Tf index is the ratio between disialo- and tetrasialotransferrin, whereas ion-exchange chromatography-nephelometry measures all of the desialylated transferrin fractions collected in the eluate. Few studies have included a correlation study between two different methods. Two studies reported approximately the same correlation [25, 46] as our study, and Lof et al. observed a very strong correlation between a chromatographic technique and a technique based on isoelectric focusing [28]. These variations between methods occur because different methods do not measure the same transferrin fractions and because there is a nonproportional increase in the different fractions that constitute CDT. Moreover, CDT values are sometimes expressed as an absolute amount of some transferrin fractions and sometimes as a relative amount (relative to total transferrin or to a major fraction). Using the relative measurement does not appear to improve the reproducibility of results over the simple measurement of CDT [29, 47], at least partly because of individual variations in serum transferrin independent of alcohol consumption. When we calculated the relative value of CDT as a percentage of total transferrin, neither diagnostic characteristics nor correlation with Tf index was improved.

**Clinical Application**

Other chromatographic techniques have given various values for control populations [6, 12, 15, 18, 25], ranging from 20 U/L (or mg/L) to 78 U/L (or mg/L). These differences generally are caused by the chromatographic step, which is dependent on several factors (e.g., gel, buffer, proportion between height and diameter of the column, iron saturation). Generally, the greater the proportion of disialotransferrin that is eluted, the higher the CDT values. This is why the commercial kit CDToct (Kabi Pharmacia, Uppsala, Sweden), which measures mainly asialotransferrin, reports low concentrations of CDT. The buffer used in the CDToct kit has a higher pH and a higher ionic strength than the buffer we used. These differences lead to a higher recovery of disialotransferrin in the method described here.

We have no explanation for the high concentrations (90–129 mg/L) observed in some individuals in the control group. Perhaps the distribution of CDT concentrations was very asymmetric in our controls, or perhaps the alcohol consumption of some control subjects was considerably underestimated. Isoelectric focusing showed that these results were not due to defective determinations or to genetic variants. Individuals with D-variant transferrin produce some false-positive results for CDT [41, 43, 44], but this genotype is present in <1% of the Caucasian population [32]. Patients with B-variant transferrin may frequently be falsely negative for CDT, but none was in this study. In our experience, the prevalence of such B-variants is <0.5%.

As in other studies [6, 15, 35], we were not able to establish a correlation between alcohol consumption at admission and CDT concentration. This is not really surprising for at least two reasons: The evaluation of alcohol consumption was not precise, and the exact mechanism by which alcohol abuse increases CDT is not fully understood. The upper limit of normal values was set at 70 mg/L to obtain a specificity of 0.90; as the inset to Fig. 4 shows, a higher limit would lead to a decrease in sensitivity more marked than the gain in specificity. Even so, the 70 mg/L cutoff resulted in lower sensitivity than has been reported in some other studies [6, 10, 12, 13, 17, 33], whereas others have reported equal or lower sensitivity [11, 15, 18, 25]. It is difficult to know whether these differences reflect a lack of sensitivity by the present method in comparison with the chromatography–RIA method or are due to differences in the population samples.

The analytical specificity of a test is its ability to determine exclusively the analyte that is supposed to be measured. In the present method, the nephelometric assay measures only transferrin, so the specificity is determined by only the chromatographic step. Given that the eluates were shown by isoelectric focusing to be free of tri-, tetra-, and pentasialotransferrin, we think the method may be considered specific. The diagnostic specificity is the fraction of nonalcohol abusers whose CDT value is within the normal range, regardless of any pathological condition. In the present study, the specificity was calculated from results obtained in a population of healthy subjects. Moreover, in many studies, the specificity for CDT measured by different methods remained high in various pathological conditions [6, 9, 12, 24, 25, 35, 36, 38, 45, 46, 48–50]. Thus, it is very likely that the present method has the same rate of false positives as the other ones.

Most studies [3, 6, 9–13, 15, 18, 51, 52] have reported about the same specificity as the present study, which can be explained by the choice of an appropriate cutoff value. A low specificity has been reported in hypertensive patients [48], but the relationship between CDT and hypertension has not been established [53]. Because CDT concentrations >100 mg/L in control subjects are very unusual, therefore, a CDT concentration >70 mg/L indicates probable alcohol abuse (specificity = 0.90). For CDT concentrations >100 mg/L, the probability of alcohol abuse is very high, the specificity with this cutoff value being 0.99.

In conclusion, we have developed an alternative to ion-exchange–RIA for measuring CDT. The discrimination between
alcoholics and control subjects by our method is at least as good as by isoelectric focusing. Compared with the ion-exchange–RIA method, the ion-exchange-nephelometry method permits determinations of CDT by laboratories that lack equipment for radioisotopes. This technique does not require the establishment of a calibration curve for each run and allows individual testing, unlike ion-exchange–RIA or ion-exchange–enzyme immunoassay methods.

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