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CARBOHYDRATE-DEFICIENT TRANSFERRIN MEASURED BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND CDTect™ IMMUNOASSAY

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Abstract — Carbohydrate-deficient transferrin (CDT) was measured in three populations using anion-exchange chromatography followed by radioimmunoassay (CDTect™) and a new high-performance liquid chromatography (HPLC) method. The correlation between the methods in 50 consecutive clinical samples was good (r = 0.87). However, in a set of 49 samples with CDT concentrations close to the reference value of the methods, the correlation was low (r = 0.51). In addition, among controls, no correlation between the methods was found (r = 0.10). Abnormal isoforms of transferrin were noted in 5% of the clinical samples. HPLC determination of CDT offers the advantage of identifying these isoforms of transferrin and thus reducing analytical pitfalls associated with the CDTect™ method.

INTRODUCTION

Carbohydrate-deficient transferrin (CDT) has emerged as a specific marker of alcohol abuse (for review see Stibler, 1991; Allen et al., 1994; Goldberg and Kapur, 1994; Rosman and Lieber, 1994). Several methods have been developed for the analysis of CDT. Originally, transferrin isoforms were analysed using isoelectric focusing (Stibler et al., 1978). Anion exchange chromatography followed by radioimmunoassay (CDTect™) has become the dominant commercially available method (Stibler et al., 1986). However, other methods have also been developed and evaluated (Storey et al., 1985; Anton and Bean, 1994; Bell et al., 1994a; Sillanaukee et al., 1994). Recently, a method based on ion exchange chromatography using a high-performance liquid chromatography (HPLC) technique has been developed (Jeppsson et al., 1993). The aim of this study is to evaluate the HPLC method in relation to commercially available ion-exchange chromatography.

MATERIALS AND METHODS

Subjects

To establish reference values for the HPLC method, 88 healthy blood donors (61 men and 27 women) were anonymously asked to state their alcohol intake during the week prior to sampling. Mean alcohol intake was 61 g/week (median = 40 g/week, range = 0–225 g/week). Samples from 48 of these subjects (32 men and 16 women) were stored at −20°C and later analysed using CDTect™. Written informed consent was obtained from control subjects.

To compare the methods in a clinical setting, the two methods were used to analyse two sets of routine samples received at the laboratories for analysis. The first set consisted of 50 consecutive samples, whereas the second consisted of 49 samples from male patients found to have CDT levels of 10–30 U/l according to CDTect™. This second set of samples was chosen to study further the correlation of the methods near the reference limit.

Methods

HPLC analysis was performed according to
Jeppsson et al. (1993). Serum was iron-saturated by adding 25 μl of 0.5 mol/l NaHCO$_3$ and 20 μl of 10 mmol/l FeCl$_3$ to 500 μl of serum. Samples were mixed and kept at 8°C overnight. Lipoproteins were precipitated by adding 50 μl of magnesium dextran solution (2 mol/l MgCl$_2$ + 20 g/l dextran sulphate). Samples were incubated for 15 min at 8°C and centrifuged (1500^ for 30 min). Two hundred μl of the supernatant were transferred to vials and diluted with 400 μl of H$_2$O.

HPLC separation was performed using a Hewlett Packard HPLC 1050 system (Hewlett Packard, Avondale, PA, USA) using Mono Q HR 5/5 column (Pharmacia, Uppsala, Sweden) with a flow rate of 1 ml/min. Detection was done at 460 nm. Gradient A: 20 mmol/l Bis-Tris; gradient B: 20 mmol/l Bis-Tris with 350 mmol/l NaCl; gradient D: 2 mol/l NaCl; gradient C: 0.02% sodium azide. Bis-Tris was from Sigma, St Louis, MO, USA and dextran sulphate was from Pharmacia, Uppsala, Sweden. All other chemicals were from Merck, Darmstadt, Germany. Gradient profile and regeneration of the column were as described by Jeppsson et al. (1993). Integration was done automatically according to the valley-valley method and each transferrin isoform was expressed as % of total transferrin area. CDT was calculated as the area of peaks with pI 5.7 and, when present, pI 5.9. Total coefficient of variation (CV) was 8% in 40 controls run on one column (mean CDT in control sample = 1.63%, range = 1.40–1.97%) and 12% in 53 controls run on two different columns (mean CDT in control sample = 1.57%, range = 1.05–1.91%). When 19 different samples, spanning a wide range of CDT levels, were analysed in duplicate the mean intra-assay CV was 8%.

CDTect™ was performed according to instructions from the manufacturers as described previously (Hultberg et al., 1995). Serum was ion-saturated and CDT quantified by a double-antibody radioimmunoassay utilizing a commercial kit, CDTect™ (Pharmacia, Sweden). Inter- and intra-assay CV was <10%.

Total serum transferrin concentrations were determined immunoturbidimetrically using Transferrin Tina-Quan® on Hitachi 911 (Boehringer, Mannheim, Germany) calibrated against CRM470 using Human Serum Protein Calibrator X 908 (from DAKO, Copenhagen, Denmark).

RESULTS

Serum CDT measured by HPLC in 88 control subjects with low or moderate ethanol consumption was 0.54 ± 0.26% (mean ± SD, median = 0.52%, range = 0.1–1.2%). No significant sex difference was found (men = 0.53%, women = 0.58%). Based on these results, the reference interval was set at ≤1%. When 48 of these samples were analysed using CDTect™, CDT was 14.2 ± 3.8 U/l (median = 13.0 U/l, range = 9–26 U/l). No sex difference was noted (men = 14.2 U/l, women = 14.4 U/l). One male control subject had elevated CDT level (26 U/l) despite reporting no alcohol consumption during the week before investigation. All other results were below the reference values recommended by the manufacturers of CDTect™ (<20 U/l and <26 U/l for men and women, respectively). In this control population, no correlation was found between results obtained by the two methods (r = 0.10, Fig. 1). Regardless of method used, no significant correlation was found between CDT levels and alcohol consumption, age or serum transferrin levels (data not shown).

In order to compare the HPLC method with CDTect™ over a wide range of values, 50 clinical samples analysed by the HPLC method were also tested with the CDTect™ method. A correlation coefficient of 0.87 was obtained (P < 0.001, Fig. 2). Using the established reference values, four
subjects (8%) in this population had CDT levels above the reference interval for the CDTect™ method, while having normal values when analysed with the HPLC method. In two cases (4%), elevated levels were found on the HPLC method while normal concentrations were found using CDTect™.

Serum transferrin was weakly correlated to CDTect™ levels \( (r = 0.28, P < 0.05) \) but not to CDT levels as measured by HPLC \( (r = -0.05) \). A ratio of CDTect™ to serum transferrin was calculated. The correlation between the CDTect™/transferrin ratio and CDT measured by HPLC was 0.93.

Regarding discrepancies between the methods, five cases were of particular interest. In two of these, CDTect™ showed markedly elevated concentrations (26 and 37 U/l) while normal values were obtained by HPLC (0.6 and 0.5%, respectively). In the other three cases, presumably genetic variations of transferrin isoforms were noted (Fig. 3). Discrepant results were found in two of these cases. In one case (B) the genetic variation made it impossible to separate CDT with \( \text{pl} = 5.7 \) from the transferrin isoform with \( \text{pl} = 5.6 \). In the CDTect™ assay, this patient had highly elevated concentration of CDT (47 U/l). To clarify if this chromatographic pattern was caused by inadequate separation of the two peaks, samples were reanalysed, but with an identical result. In several cases, new samples were obtained and found to have the same chromatographic pattern. In case C with two major peaks, the HPLC value was elevated, whereas CDTect™ was normal. It is noteworthy that this patient also had a very low concentration of total serum transferrin (1.4 g/l). In the last of these cases, a similar pattern as in case C was noted. In this sample, slightly elevated CDT levels (1.1% and 22 U/l) were found by both methods.

To study further the correlation between the HPLC and CDTect™ method, 49 samples from male subjects having CDTect™ values near the reference point were selected (Fig. 4). In this range, the correlation between the methods was markedly lower than in the set of samples with a wide range of CDT values \( (r = 0.51, P < 0.001) \). In this population, there was no correlation between serum transferrin and CDT. As was the case in the previous two series of samples, a number of discrepancies between the methods were observed. In seven cases (14%) a clear difference was found between the results of the two methods. In six of these samples, elevated CDT values were obtained with the CDTect™ method while normal levels were found using HPLC. Only one case was found where the HPLC method provided raised CDT level while the result from CDTect™ was normal. Two cases of abnormal variants of transferrin were found. The chromatographs of these samples were similar to the chromatograph in Fig. 3B. In these two cases, it was not possible to quantify CDT, as the CDT peak corresponding to \( \text{pl} 5.7 \) comigrated with the 5.6 peak.

**DISCUSSION**

In this study, a new HPLC method for the determination of CDT was compared to the commercially available CDTect™ kit. The overall correlation between the two methods was acceptable, especially when comparing samples spanning a wide range of CDT levels. These results are thus in accordance with previous methodological studies comparing CDTect™ with other techniques (Anton and Bean, 1994; Sillanaukee et al., 1994). However, the correlation is less convincing at lower levels of CDT, a finding which is in line with the data presented by Anton and Bean (1994). In addition, no significant correlation between the methods was found in subjects consuming low to
Fig. 3. Chromatographic profiles of different isoforms of transferrin found after HPLC separation. 
(A) Normal profile. (B) Isoform where CDT with pl 5.7 eluted with transferrin isoform with pl 5.6. (C) Isoform with two major peaks. CDT with pl 5.7 was eluted after 9.1–9.2 min. (D) Sample with normal isoforms of transferrin and high CDT level (6.4%). Arrows indicate CDT with pl 5.7 and 5.9.
moderate amounts of ethanol. A weak correlation was observed when samples close to the reference range of CDT were analysed using the two methods. The lack of correlation is most likely the reason for the large number of discrepancies found in these samples with concentrations close to the reference limit. In all, 13% of the samples were classified differently with the two methods. Of these samples, most had elevated CDTect™ levels while the HPLC values were normal. As the alcohol consumption in this group was not known, it is not possible to state if the CDTect™ method is more sensitive or, if not, is less specific for detecting alcohol abuse.

It is important to note the analytical variability of the two methods studied. With coefficients of variation of ~10%, a considerable degree of uncertainty exists. This is particularly important when interpreting results close to the reference value. The setting of a valid reference range is also of importance. In the original publication on the HPLC method, the reference range was set at <0.8% (Jeppsson et al., 1993). In our material, the value under which results from the control population were found was ≤1.0%. A similar reference range has been suggested by Seitz et al. (1995). Recently, a reference range of <1.2% has been recommended on the basis of a population study on 250 persons in southern Sweden. These variations are expected as the HPLC assay is presently in its early stage of clinical implementation. With regard to reference range, it is also necessary to consider that the CDT level is a continuous variable with no clear-cut value at which the result can be definitely considered to be a marker for alcohol consumption of a given amount.

A number of presumably genetic variations of transferrin were found when CDT was analysed using HPLC. To eliminate the possibility that methodological factors caused these chromatographic patterns, samples were reanalysed with identical results. When new samples from patients with these isoforms were analysed, a similar chromatographic pattern was obtained. The finding of isoforms of transferrin is in line with the reported polymorphism of the protein (Kamboh and Ferrell, 1987; Bean and Peter, 1994). These abnormal forms of transferrin, which may give rise to inaccurate determinations of CDT, cannot be detected with CDTect™. These variations were present in ~5% of the samples. The exact prevalence of these variations cannot be stated with certainty as more material will be needed to make such an estimation. The most obvious advantage of the HPLC method is that these isoforms can be readily detected. Therefore, with the use of the HPLC method, analytical pitfalls can be avoided in a substantial number of clinical samples. However, it is noteworthy that reliable determination of CDT by HPLC cannot be obtained in these cases.

CDT is expressed as a percentage of total transferrin in the HPLC method and as units per volume with the CDTect™ method. As serum transferrin varies, this may be one reason for discrepancies observed between the two methods. This was also the case in some, but not all, samples with high plasma concentrations of transferrin. However, expressing the CDTect™ results as a ratio to serum transferrin did not significantly improve the correlation between the methods studied. Similar conclusions have been drawn by some, but not all, investigators when trying to improve the performance of the CDTect™ method (Behrens et al., 1988; Schellenberg et al., 1989; Sillanaukee et al., 1994; Bell et al., 1993, 1994b).

In conclusion, an acceptable overall correlation was found between CDTect™ and the HPLC method for the determination of CDT. However,
the HPLC technique offers advantages, especially as it can identify genetic variations of transferrin and thus avoid incorrect estimations of CDT.

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