

take extreme care in specimen handling and storage, as well as in randomization of specimen preparation and spectrum collection times, to discover true disease-related spectral profiles.

We thank Ingrid Pollet and Fred Wong for assistance with serum sample freezing. This study was funded by grants to A.K. from the National Cancer Institute of Canada with funds from the Canadian Cancer Society and to A.K. and K.G. from the Canadian Breast Cancer Foundation (BC Chapter), and in part with US funds from the National Cancer Institute, National Institutes of Health, under Contract NO1-CO-12400 to T.V., T.P.C., and Z.X. A.K. is supported by a personnel award from the Heart and Stroke Foundation of Canada and a Scholarship from the Michael Smith Foundation for Health Research.

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Previously published online at DOI: 10.1373/clinchem.2005.050708

Comparison of HPLC and Capillary Electrophoresis for Confirmatory Testing of the Alcohol Misuse Marker Carbohydrate-Deficient Transferrin, Anders Helander,^{1*}

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The major form of the iron-transport glycoprotein transferrin in blood contains 2 N-linked disialylated biantennary oligosaccharide chains (glycans) and is named tetrasialotransferrin. Regular high alcohol consumption (mean of at least 50–80 g/day) generally alters the glycosylation profile of transferrin (1), increasing the relative amounts of glycoforms lacking one (disialotransferrin) or both (asialotransferrin) N-glycans (2, 3). The alcohol-related glycoforms are collectively referred to as carbohydrate-deficient transferrin (CDT). CDT measurements are widely used for identifying individuals with alcohol problems in various medical settings (e.g., addiction treatment) and for monitoring abstinence from alcohol in outpatient treatment programs (e.g., when drunk-driving offenders reapply for a driver's license) (4). When drinking is discontinued, the CDT concentration normalizes with a half-life of 1.5–2 weeks (5, 6). The main advantage of CDT over the conventional alcohol biomarkers, such as the liver function test γ -glutamyltransferase, is the higher specificity for alcohol misuse with resulting lower risk for false-positive identifications (7, 8).

Since the discovery of CDT as an alcohol marker (1), a multitude of analytical techniques and methods have been applied for its measurement (1, 9). The most widely used assays worldwide today are the Axis-Shield %CDT immunoassay and various automated applications thereof, such as %CDT TIA from Bio-Rad and Tinaquant® %CDT from Roche (10, 11). These assays are based on ion-exchange minicolumn chromatographic isolation of the CDT fraction, separate measurement of CDT and total transferrin using the same transferrin antibody, and calculating CDT as a percentage of total transferrin (%CDT). Immunologic methods are convenient and time-efficient for routine use in central laboratories with high specimen throughput, but because these tests separate CDT from non-CDT moieties on the basis of differences in isoelectric point (pI), they will be disturbed by genetic transferrin polymorphisms (12) and by congenital disorders of glycosylation (13), which can cause falsely high or low results that may lead to false-positive or -negative identification of patients for alcohol misuse. Accordingly, when the %CDT ion-exchange immunoassay combination is used in medico-legal cases, such as traffic medicine (14, 15), there is a need to confirm the test result by an independent separation method to rule out analytical interferences as the cause of a high value (12, 16, 17).

This study compared the performances of 2 laboratory

methods for confirmatory and routine testing of %CDT in serum, HPLC and capillary electrophoresis (CE). Both methods produce a fingerprint of the glycoform distribution and allow for quantitative and reproducible determination of single transferrin glycoforms. We compared the results with those obtained by a %CDT minicolumn immunoassay (Axis-Shield %CDT assay).

The 79 sera used in this study were selected from the routine samples pool to cover the range from low-normal to highly increased %CDT values (1.3%–24.2% by the Axis-Shield %CDT assay) as well as some genetic transferrin variants. At the Karolinska University Hospital in Stockholm, 42 anonymous surplus sera were collected and randomly analyzed in single determinations for transferrin glycoforms by HPLC. A portion of each of these specimens was sent frozen to the Meander Medical Center in Amersfoort for blinded CE analysis, also using single determinations. Another 37 serum samples were collected in Amersfoort, analyzed by CE, and sent to Stockholm for blinded HPLC analysis. Serum was separated by centrifugation, and samples were stored at -20°C until analysis.

HPLC separation of transferrin glycoforms was performed on a SOURCE[®] 15Q anion-exchange column (Amersham Biosciences) by linear salt gradient elution, and quantification relied on measurement of the absorbance of the iron-transferrin complex at 470 nm (17). The relative

amounts of single glycoforms to total transferrin were calculated as percentages of peak areas with use of baseline integration. The CE method was the CEofix[™] CDT assay (version improved in November 2003; part no. 10-004260 Europe 844111046) from Analis (Belgium). The samples were iron-saturated, and electrophoresis was carried out with ultraviolet detection at 214 nm on a Beckman Coulter P/ACE 5000, according to the manufacturer's instructions. The relative amounts of single transferrin glycoforms were calculated from peak areas by valley-to-valley integration, as suggested by the manufacturer. The %CDT was measured immunologically in single determinations by use of the Axis-Shield %CDT assay according to the manufacturer's instructions. This assay measures the sum of CDT glycoforms with a $\text{pI} > 5.65$ (i.e., mainly asialo-, monosialo-, and disialotransferrin) relative to the total amount of transferrin in the serum sample.

The HPLC and CE methods allowed for reproducible separation and quantification of single transferrin glycoforms with similar peak patterns. When analysis was performed on a transferrin-free serum sample obtained by immunosubtraction with rabbit anti-human transferrin (17), no interfering peaks were detected by either method (data not shown), thus indicating that the peaks observed in the HPLC chromatogram and CE electropherogram were indeed transferrin glycoforms. Shown in Fig. 1A are examples of a typical HPLC chromatogram and the cor-

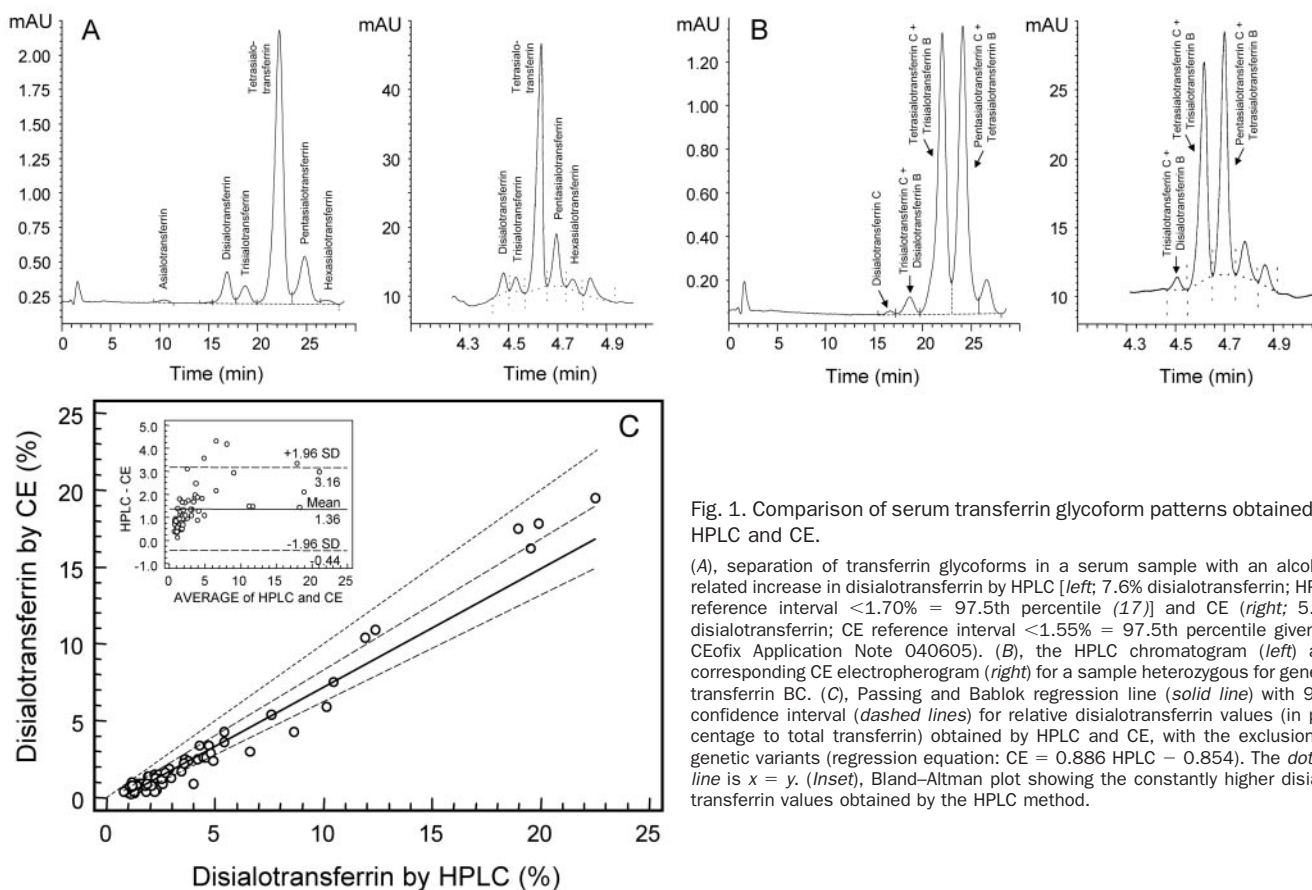


Fig. 1. Comparison of serum transferrin glycoform patterns obtained by HPLC and CE.

(A), separation of transferrin glycoforms in a serum sample with an alcohol-related increase in disialotransferrin by HPLC [left; 7.6% disialotransferrin; HPLC reference interval $<1.70\%$ = 97.5th percentile (17)] and CE (right; 5.4% disialotransferrin; CE reference interval $<1.55\%$ = 97.5th percentile given in CEofix Application Note 040605). (B), the HPLC chromatogram (left) and corresponding CE electropherogram (right) for a sample heterozygous for genetic transferrin BC. (C), Passing and Bablok regression line (solid line) with 95% confidence interval (dashed lines) for relative disialotransferrin values (in percentage to total transferrin) obtained by HPLC and CE, with the exclusion of genetic variants (regression equation: $\text{CE} = 0.886 \text{ HPLC} - 0.854$). The dotted line is $x = y$. (Inset), Bland-Altman plot showing the constantly higher disialotransferrin values obtained by the HPLC method.

responding CE electropherogram for a serum sample with an alcohol-related increase in the relative amount of disialotransferrin and a visible asialotransferrin by HPLC. Rare genetic transferrin variants and glycoform types, including transferrin B homozygotes and BC and CD heterozygotes, another variant tentatively identified as "C2C3" (12), and serum samples containing high relative amounts of monosialo- and trisialotransferrin, all identified as actual or potential causes of falsely high or low %CDT results with the minicolumn immunoassays (12), were readily identified by both methods. Examples of an HPLC chromatogram and the corresponding CE electropherogram for a sample heterozygous for the genetic transferrin BC are shown in Fig. 1B.

The relative amounts of disialotransferrin, the major glycoform in CDT, to total transferrin (i.e., total peak area for all transferrin glycoforms) obtained by HPLC and CE were highly correlated ($r^2 = 0.972$; $P < 0.0001$; Fig. 1C). However, the HPLC method constantly yielded higher results, the values being 1.36% higher, on average (range, 0.15%–4.20%; $P < 0.0001$), than the CE values ($n = 68$; genetic variants were excluded from these calculations; Fig. 1C, inset). Use of the baseline integration mode for a subset ($n = 32$) of the CE results, instead of the valley-to-valley integration mode recommended with the CEofix CDT assay, only partly compensated for this difference; the HPLC disialotransferrin values were still 0.97% higher, on average (range, –0.01% to 2.75%; $P < 0.0001$).

The %CDT results obtained with the minicolumn immunoassay were highly correlated ($r^2 = 0.904$; $P < 0.0001$) with the corresponding HPLC results (i.e., sum of asialo-, monosialo-, and disialotransferrin). However, several samples with genetic transferrin variants (e.g., transferrin CD and C2C3) produced falsely high %CDT results with the Axis-Shield %CDT assay (data not shown), which agrees with previous observations (12). It should be noted that the first direct immunoassay for %CDT (N Latex CDT; Dade Behring) will soon be commercially available. This new method is based on a monoclonal antibody that measures specifically the transferrin glycoforms lacking 1 or 2 N-glycans and on a simultaneous total transferrin assay (18). Accordingly, genetic transferrin variants B and D, albeit having primary structures different from that of the common transferrin C phenotype but a normal set of carbohydrate chains, are indicated not to interfere with the %CDT measurement by the N Latex CDT (19), which represents a major improvement over the minicolumn versions in terms of higher test specificity.

The present results show that the HPLC and CE methods for %CDT allow for visible and reproducible determination of transferrin glycoforms in small sample volumes. Most importantly, the genetic transferrin variants and glycoform types that often cause falsely high or low results with the current %CDT minicolumn immunoassays were readily identified by both methods. The relative amounts of disialotransferrin obtained by HPLC and CE were highly correlated, but the HPLC method constantly yielded higher values. One possible reason for this difference was related to the integration modes used for quan-

tification of transferrin glycoform peak areas (baseline for HPLC and valley-to-valley for CE), but use of baseline instead of the recommended valley-to-valley integration with the CE method only partly compensated for this difference. It might be that the CE method used (CEofix CDT assay) is less sensitive for quantification of transferrin than the HPLC method, which agrees with previous results (17, 20). This could result from the CE method relying on nonspecific measurement of the peptide bond, whereas the HPLC method measures the selective absorbance of the transferrin-iron complex. However, improved CE methods with better resolution between disialo- and trisialotransferrin and thus producing results for the CDT glycoforms approaching those obtained by HPLC, have recently been published (21, 22).

In summary, these results support the use of HPLC and CE methods for confirmation of %CDT immunoassay results in medico-legal cases and for evaluation of borderline values. Several commercial HPLC and CE assays for %CDT testing with high capacity and reduced total analysis time are already, or will soon be, commercially available.

This work was supported by grants from the Karolinska Institutet (to A.H.).

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DOI: 10.1373/clinchem.2005.050732

β -Trace Protein Is an Alternative Marker for Glomerular Filtration Rate in Renal Transplantation Patients, Uwe Pöge,^{1*} Thomas M. Gerhardt,¹ Birgit Stoffel-Wagner,² Holger Palmedo,³ Hans-Ulrich Klehr,¹ Tilman Sauerbruch,¹ and Rainer P. Woitas¹ (Departments of ¹Medicine, ²Clinical Biochemistry, and ³Nuclear Medicine, University of Bonn, Bonn, Germany; * address correspondence to this author at: Medizinische Klinik und Poliklinik I der Universität Bonn, Allgemeine Innere Medizin, Sigmund-Freud-Strasse 25, D 53105 Bonn, Germany; fax 49-228-287-4952, e-mail u.poege@uni-bonn.de)

Careful monitoring of graft function after renal transplantation (RTx) is necessary for short- and long-term success. Because of the many difficulties in performing serial measurements of glomerular filtration rate (GFR) by use of exogenous substances such as inulin, ⁵¹Cr-EDTA, or technetium-diethylenetriaminepentaacetic acid (^{99m}Tc-DTPA), graft function is often estimated from serum creatinine concentrations. Tubular secretion as well as various nonrenal factors may alter creatinine concentrations, thus rendering it an imprecise marker for GFR (1). Cystatin C (Cys C) is at least as good as creatinine as a marker of renal function (2–4), but corticosteroid treatment can alter serum Cys C concentrations (5–7), making it potentially less useful in RTx patients.

β -Trace protein (BTP), also known as prostaglandin D synthase, is a 23- to 29-kDa enzyme that has been proposed as an alternative marker for GFR in children and in persons with diabetes or various renal diseases (8–11), but it has not been examined as an endogenous renal function marker in RTx patients. The aim of this study

was to compare the diagnostic performances of BTP, Cys C, and creatinine as estimates of GFR in RTx patients.

We prospectively performed GFR measurements in 85 consecutive RTx patients (34 females and 51 males) who attended our outpatient department. GFR was determined by ^{99m}Tc-DTPA clearance with a single-injection technique based on the method described by Russell et al. (12).

Patients included into the study were in “steady-state” conditions, defined as the lack of increases or decreases >15% in creatinine within 2 weeks before and after the investigation.

The mean (SD) patient age was 49.6 (13.3) years (range, 19–72 years), and the time frame of investigation was 75.7 (71.2) months after transplantation (range, 3–240 months; 21 patients within 12 months after RTx, 39 patients at 1 to 10 years after RTx, and 25 patients more than 10 years after RTx).

Immunosuppression was based on a calcineurin inhibitor regimen in 83 patients (51 on cyclosporin A, 32 on tacrolimus). Of these, 81 patients were treated with corticosteroids, and 42 patients also received mycophenolate-mofetil (n = 40) or azathioprine (n = 2). One patient was on sirolimus, mycophenolate-mofetil, and corticosteroids, and another was on sirolimus, tacrolimus, and corticosteroids.

The study was approved by the local ethics boards and was performed in accordance with the ethics guidelines of the revised Helsinki Declaration of 1996. Informed consent was obtained from all patients.

Creatinine, BTP, and Cys C concentrations were measured simultaneously. Serum creatinine was measured by a modification of the Jaffe method according to Knapp and Mayne (13). Cys C and BTP were measured by fully automated latex-enhanced immunonephelometric methods (N Latex Cystatin C and N Latex beta-trace protein; Dade Behring) on a Behring Nephelometer II according to the manufacturer’s instructions (9, 10). Bias was 2.55% (mean, 1.06 mg/L; n = 20) for the Cys C assay and 2.35% for the BTP assay (mean, 1.49 mg/L; n = 20). The within-run imprecision (CV) for the Cys C assay was 3.4% at 1.09 mg/L (n = 20), and the between-run CV was 4.7% at 1.06 mg/L (n = 20). Corresponding values for the BTP assay were 3.6% (at 1.47 mg/L; n = 20) and 4.6% (at 1.49 mg/L; n = 20).

Reference intervals established from a representative cohort of 200 healthy blood donors [100 female; median age, 31 years; 95% confidence interval (CI), 19.0–60.5 years] were 0.48–0.82 mg/L for Cys C, 0.40–0.74 mg/L for BTP, and 53–106 μ mol/L for creatinine.

Results are presented as the mean and 95% CI unless indicated otherwise. Statistical analysis was performed with Medcalc[®] (Ver. 4.2) and StatView[®] (Ver. 5.0 for Windows; SAS Institute Inc.). *P* < 0.05 (2-tailed) was considered significant.

The mean (95% CI) GFR was 38.6 (35.3–41.9) mL \cdot min^{–1} \cdot (1.73 m²)^{–1} in our cohort, and the mean (95% CI) concentrations of plasma BTP, creatinine, and Cys C