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Toward standardization of carbohydrate-deficient transferrin (CDT) measurements: III. Performance of native serum and serum spiked with disialotransferrin proves that harmonization of CDT assays is possible

Abstract

Carbohydrate-deficient transferrin (CDT) is a generic term that refers to the transferrin glycoforms whose concentration in blood is temporarily increased by sustained alcohol consumption. Due to high clinical specificity, CDT was proposed as a biomarker of heavy alcohol use and has been available for about 20 years. A number of methods have been developed for CDT measurement based on different analytical techniques and principles and without any harmonization or calibration to a reference method. As a consequence, neither the reference limits nor the cut-off values have been similar across assays, hampering understanding of the diagnostic value of CDT and its routine use. This prompted the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) to initiate a Working Group on Standardization of CDT (WG-CDT). This third publication of the WG-CDT is devoted to testing the commutability of native and disialotransferrin-spiked serum panels as candidate secondary reference materials, in order to prove the harmonization potential of commercial CDT methods. The results showed that assay harmonization reduced the inter-laboratory imprecision in a network of reference laboratories running the HPLC candidate reference method. In the seven commercial methods evaluated in this study, the use of multi-level secondary calibrators of human serum origin significantly reduced the between-method imprecision. Thus, harmonization of CDT measurements by different methods can be achieved using this calibration system, opening the way for a full standardization of commercial methods against a reference method by use of certified reference materials.

Keywords: calibrator; carbohydrate deficient transferrin (CDT); commutability; disialotransferrin; harmonization; HPLC.

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Introduction

Carbohydrate-deficient transferrin (CDT) is a generic term that refers to the transferrin glycoforms whose concentration in blood is temporarily increased by sustained alcohol consumption [1]. Transferrin consists of a single polypeptide chain with 679 amino acids with two N-linked complex oligosaccharide chains. The microheterogeneity in the oligosaccharide chains is important: each of the two chains can be bi-, tri- or rarely tetra-antennary; each antenna usually bears a sialic acid residue at its end and the glycoform is named by the total number of sialic acid

residues [2]. The main component of serum transferrin, tetrasialotransferrin, consists of two biantennary chains and makes up approximately 80% of the total. Other glycoforms usually present in normal samples are penta-sialotransferrin (approx. 15%), trisialotransferrin (approx. 4%–5%), disialotransferrin (approx. 1.0%–1.5%) and hexasialotransferrin (approx. 1%) [3, 4].

An alcohol consumption averaging at least 50–60 g/day for more than 2 weeks is considered necessary to induce a relative increase of disialotransferrin. When the disialotransferrin level reaches approximately two- to three-fold the upper limit of the reference values, asialotransferrin usually becomes measurable [5]. Due to the high specificity for alcohol increasing disialotransferrin, CDT was proposed as a biomarker of heavy alcohol use and has been widely used as such. Over the last 20 years, a number of CDT methods were developed based on different analytical techniques and principles, however, without any standardization of the reference intervals and cut-offs. Unfortunately, a clear unifying definition of the measurand (“CDT”) has been hard to give, due to the different principles of methods employed. Initially, the sum of all glycoforms with a $pI \geq 5.7$, corresponding to disialotransferrin and the rare mono- and asialotransferrin, was referred to as CDT [1]. Some older methods also included part of trisialotransferrin. More recently, a direct immunoassay based on a monoclonal antibody that recognizes the transferrin glycoforms lacking at least one of the glycan chains was developed [6]. In the latter case, the measurand is probably a mixture of disialo-, monosialo- and asialotransferrin.

As a consequence, neither the reference limits nor the cut-off values were similar across assay methods, which hampered understanding of the diagnostic value of CDT and its routine use. This prompted the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) to initiate a Working Group aiming for standardization of CDT measurements (WG-CDT). In its first publication [7], disialotransferrin (disialylated monoglycan transferrin) was defined as the measurand for a reference method and the target analyte for the standardization work, and high-performance liquid chromatography (HPLC) [8] with photometric detection was proposed as the candidate reference method. A network of reference laboratories running this method was formed demonstrating good intra- and inter-laboratory performance [9].

Recently, the WG-CDT focused their efforts on the development of secondary reference materials to be used by the manufacturers to standardize their test results to the reference method. These materials must be commutable, to allow harmonization of all different methods currently in use, and be stable in storage.

The present study investigated the suitability of native and disialotransferrin-spiked serum panels as candidate secondary CDT reference material. This was done by testing the commutability in the reference laboratories, the manufacturers’ laboratories, and in two external quality assurance (EQA) schemes. Whether a single or multiple point calibration is needed for calibration of the present commercial methods was also examined.

Samples

The study was based on the use of two six-level series of samples with similar disialotransferrin concentration ranges, one being constituted from native human serum samples and the other formed by spiking a low CDT serum sample with isolated disialotransferrin.

Initially, two serum pools were prepared from anonymous leftover volumes of samples from routine examinations in abstaining patients (pool P1) and alcoholic patients (pool P6). The CDT level in these pools was determined using the HPLC candidate reference method [8] and the relative concentrations of disialotransferrin were found to be approximately 1.2% for P1 and approximately 8.0% for P6. Then, four intermediate CDT levels (P2–P5) were produced by a mathematical dilution (equal intervals) of P1 and P6 to obtain disialotransferrin concentrations of 2.6%, 3.9%, 5.3% and 6.6%. These six levels constituted the patient (P) panel.

A second panel was prepared by spiking another aliquot of the P1 pool with purified disialotransferrin, obtained by courtesy of Bio-Rad (Munich, Germany) [10], to a final disialotransferrin concentration of 8.8% (pool S6). As for the patient panel, four intermediate CDT levels (S2–S5) were prepared by dilution of P1 and S6 to obtain disialotransferrin concentrations of 2.7%, 4.2%, 5.8% and 7.3%. These six levels constituted the spiked (S) panel.

All samples were distributed into aliquots and lyophilized using the procedure described in a previous study [9]. It was found in preliminary experiments that these samples were stable for at least 2 years when stored frozen at -20°C (Weykamp and Wienders, unpublished).

Analytical methods

The commutability of the patient and spiked panels was assessed with the most frequently used commercial CDT methods, which are based on various analytical principles. Three capillary zone electrophoresis (CE) methods were included: the CEofix CDT procedure [11] from Analis

(Suarlée, Belgium) and the Capillarys [12] and Minicap methods of Sebia (Lisses, France). Four HPLC methods were included: the candidate reference method [8], the %CDT by HPLC assay of Bio-Rad (Munich, Germany) with both standard [13] and fast procedures, and the ClinRep CDT [14] of Recipe (Munich, Germany). The immunonephelometric N Latex CDT assay [6] of Siemens (Marburg, Germany) was also included.

All CDT values by HPLC and CE methods in this study are expressed as disialotransferrin percentage of total transferrin (%DST). With HPLC, %DST represents the area under the disialotransferrin peak compared to the total peak area of all transferrin glycoforms as monitored at 470 nm. This wavelength is specific for the iron-transferrin complex. The same glycoform pattern is monitored at 200–214 nm using the CE techniques, assuming no interferences from other proteins with similar electrophoretic mobility. For the N Latex CDT immunoassay (measuring transferrin glycoforms lacking one or both glycan chains), values are expressed as percentage of total transferrin, which is measured in a second separate assay.

Analytical performance with the HPLC candidate reference method

Imprecision

The imprecision of CDT determination for both six-level panels was evaluated in five laboratories of the reference laboratories network [9]. Each sample was measured in duplicate and the inter-laboratory imprecision was calculated for all levels of both panels. The mean inter-laboratory imprecision was defined as the mean coefficient of variation (CV). As shown in Table 1, the inter-laboratory agreement was typically very good both for the patient panel (CV range from 1.8% for P5 to 4.3% for P1; mean 3.0%) and the spiked panel (CV from 2.1% for S5 to 9.5% for S1; mean 4.0%). A CV higher than 5% was obtained for only one sample (S1). When combining all levels for each panel, the mean CVs were 3.0% for the patient panel and 4.0% for the spiked panel.

Linearity

Both six-level panels were prepared by mathematical dilutions (equal intervals between two consecutive dilutions), so the linearity of measurements could be checked by calculating the correlation between the panel number and the measured value for each laboratory. The coefficients of determination (r^2) were found to be higher than 0.996

Pools	Patient panel		Spiked panel		Harmonized spiked panel	
	Mean CDT, %	CV, %	Mean CDT, %	CV, %	Mean CDT, %	Mean CV, %
1	1.24	4.3	1.16	9.5	1.17	7.3
2	2.49	3.1	2.73	4.2	2.74	2.7
3	3.78	3.0	4.32	3.4	4.33	3.3
4	5.13	3.0	5.74	2.7	5.75	2.6
5	6.48	1.8	7.32	2.1	7.33	0.8
6	8.07	2.8	8.80	2.2	8.81	1.3
Average		3.0		4.0		3.0

Table 1 Inter-laboratory imprecision in carbohydrate-deficient transferrin (CDT) levels at the reference laboratories for two six-level panels of patient and disialotransferrin-spiked serum samples. CDT (%disialotransferrin) values are the means of duplicate measurements within each laboratory. Results for the spiked panel are given both before and after harmonization using the patient panel regression equations.

for the patient panel and higher than 0.993 for the spiked panel. When considering the mean value for each level, the coefficients were respectively 0.999 and 1.000 for the patient and the spiked panels. These results demonstrated that the HPLC candidate reference method shows very good linearity in the studied range with both native patient samples and disialotransferrin-spiked serum.

Harmonization potential

The regression equation between the sample number (P1–P6) and the measured disialotransferrin value obtained for each reference laboratory in the linearity study was calculated. This equation was then applied to the values obtained for the spiked panel, to obtain ‘calibrated spiked panel results’. As indicated in Table 1, the post-analytical calibration reduced the inter-laboratory variation expressed as the CV from 4.0% to 3.0%. This result indicated that: 1) calibration will reduce the inter-laboratory CV between the reference laboratories; and 2) patient and spiked panels can be used for harmonization between the reference laboratories.

Analytical performance with the commercial CDT methods

Imprecision

The same imprecision study was applied to the seven commercial CDT procedures mentioned above. The samples

were analyzed in the manufacturers' laboratories. As expected, due to the wide range of reference values and decision limits, the overall between-method CVs were high, ranging from 5.6% (P4) to 29.8% (P1) in the patient panel (mean 13%) and from 11.5% (S3 and S4) to 30.5% (S1) in the spiked panel (mean 16%). This again demonstrated the importance and need for standardization of commercial CDT methods.

Linearity

Establishing a calibration system requires that the methods provide linear results in the calibration range. The linearity of the seven commercial methods against the candidate reference method was tested for both the patient and spiked panels. The coefficient of determination (r^2) between the value assigned by the reference laboratories and the measured value for each method in both panels ranged between 0.991 (immunoassay, N Latex CDT) and 1.000 (CE, Analis) for the patient panel and between 0.997 (immunoassay, N Latex CDT) and 1.000 (CE, Analis) for the spiked panel. This excellent correlation clearly indicated that all commercial methods can be calibrated against the HPLC candidate reference method.

However, the calibration curves for the different CDT methods were not parallel (Figure 1). For the patient panel, the slope varied from 0.771 (immunoassay, N Latex CDT) to 1.356 (CE, Sebia Minicap), and for the spiked panel from 0.596 (immunoassay, N Latex CDT) to 1.142 (HPLC, Bio-Rad fast). In addition, the intercept to the origin were comprised between -0.634 (CE, Sebia Capillarys) and 1.079 (immunoassay, N Latex CDT) in the patient panel and between -1.443 (CE, Sebia Minicap) and 1.079 (immunoassay, N Latex CDT) in the spiked panel. These discrepancies are explained by the different analytical principles and calibration methods used in the commercial methods. As the calibration curves were not parallel, standardization of results between methods will require calibrators, and because the curves did not pass through the origin, multi-point calibration is necessary.

Origin of non-commutability

There are three major effects causing non-commutability of analytical results that are attributable to the sample: 1) the matrix (biological or artificial) in which the measurand is dissolved may interfere with the analytical method; 2) pretreatment (e.g., lyophilization) of the sample may cause changes in physical, chemical or immunochemical

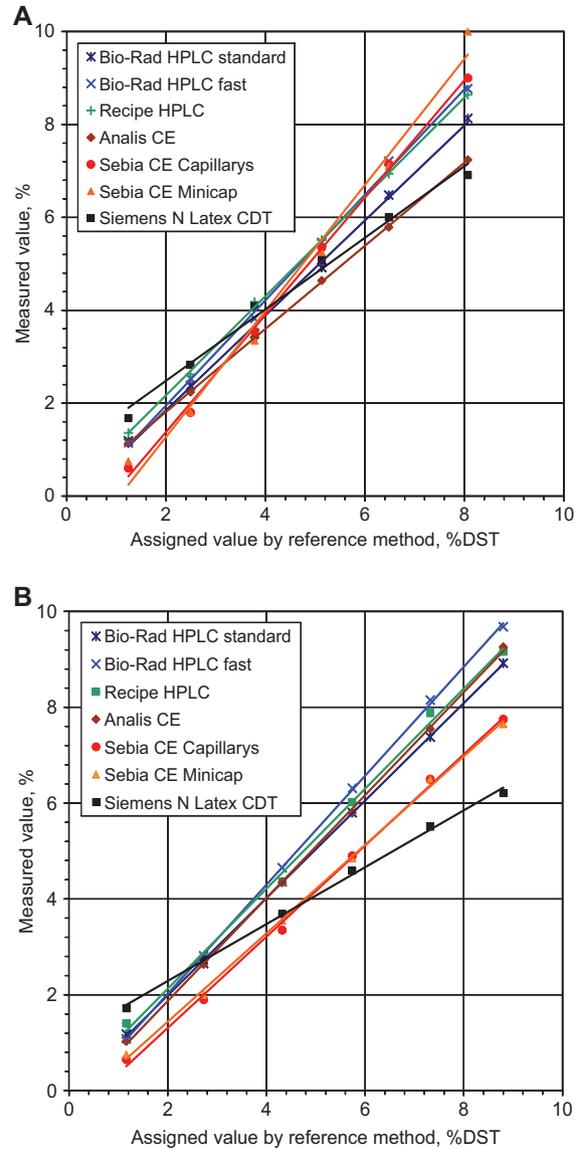


Figure 1 %CDT (N Latex CDT immunoassay) and %disialotransferrin (HPLC and CE methods) results for patient and spiked panels using commercial CDT methods plotted against the %disialotransferrin (%DST) values assigned by the network of reference laboratories using the HPLC candidate reference method. (A) Patient serum panel (P1–P6). (B) Disialotransferrin-spiked serum panel (S1–S6).

(i.e., epitope recognition) properties of the measurand; and 3) the nature of the measurand in that a native protein may not react exactly as a synthetic protein or an isolated and spiked protein, leading to a variation in the analytical result for native samples when a synthetic/isolated protein is used as calibrator. In a previous WG-CDT study [9], matrix effects due to 1) and 2) were excluded; the present study therefore focused on the nature of the measurand.

When regression (measured value vs. theoretical value) equations slopes and/or the intercept to the origin differ between native materials and synthetic materials, this can be attributed to the nature (native or synthetic) of the measurand. We calculated the difference (patient panel value – spiked panel value) using the regression equations corresponding to the data respectively presented Figure 1A and 1B for all methods. These differences are graphically presented in Figure 2. The three HPLC methods show very small differences across the range 1%–5%, while other methods are characterized by positive or negative slopes. We quantified the differences at two CDT concentrations (1.5% and 4%). At 1.5% CDT level, the differences (patient panel – spiked panel) ranged from –0.11% (immunoassay, N Latex CDT) to 0.36% (CE, Sebia Minicap). At 4%, the between analyte differences were comprised between –0.71% (CE, Sebia Minicap) and 0.41% (CE, Analis). These differences in values between the spiked and patient samples were considered as reflecting the non-commutability attributed to the nature of the measurand (Figure 2). This graph indicated that both panels are commutable for the HPLC methods, but not for the CE and immunonephelometric methods. Thus, calibration of CE and immunonephelometric methods is not possible with certified reference materials (CRM) spiked with DST; hence, the only option to prepare CRM suitable for all method principles is to manufacture them from patient samples. As these results were obtained in panels prepared by pooling several samples, a similar study will be repeated using individual patient samples following the CLSI C53A guideline.

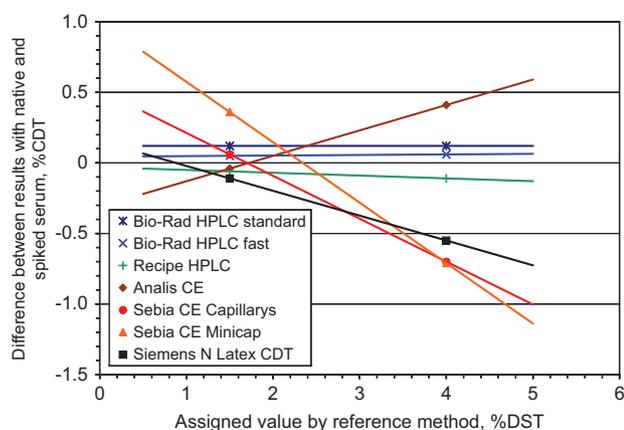


Figure 2 Non-commutability of disialotransferrin-spiked serum calibrators with the commercial CDT methods at %disialotransferrin (%DST) concentrations ranging up to 5.0%, as assigned by the network of reference laboratories using the HPLC candidate reference method.

The difference between results with native samples harmonization and spiked samples harmonization is given on the y-axis.

Calibration potential in EQA schemes

The design of the study allowed us to simulate calibration and thus to establish the harmonization potential. Using the six-level patient and spiked panels, the manufacturers established correlation equations for their CDT methods against the HPLC candidate reference method. These equations were used to recalculate the mean values from the participants of the Equalis (Sweden; n=50) and the SKML (The Netherlands; n=20) external quality assurance (EQA) programs for CDT. In this experiment, the results obtained by the laboratories of the manufacturers and the participants in the EQA schemes were merged both for the Bio-Rad HPLC (standard and fast versions) and Sebia CE (Capillars and Minicap versions) methods. For the other commercial methods, the number of participants was too low to be useful for this approach.

Three of the patient and spiked samples were used (P1, P3 and S3). Table 2 shows the high between-method CV before calculation and its substantial reduction after the introduction of the corrective equation in both native samples. On the contrary, the between-method imprecision remained high and unchanged when using the calibration regression equation provided by the spiked panel. At low levels, the between-method CV was reduced from 40% to 6% in the patient panel. At high CDT levels, the intermediate CV was reduced from 7% to 3% in the patient panel but remained high (10%–12%) in the spiked panel. This confirms that CDT harmonization can be achieved by using a multi-level panel of secondary calibrators of native (human serum) origin, but not with disialotransferrin-spiked samples.

Conclusions

These data demonstrate an excellent linearity and reproducibility of results for the HPLC candidate reference method employed by the reference laboratories. Multi-level calibration using candidate secondary calibrators marginally improved the already high inter-laboratory agreement. The commercial CDT methods gave linear results over the studied concentration range with the candidate secondary calibrators. Harmonization of results can therefore be obtained with the use of calibration by a multi-level panel of calibrators based on human serum. These promising results were observed using sample pools and have to be confirmed in a further experiment based on samples from individual patients, according to

Method	Harmonization potential					
	Patient panels P1 and P3				Spiked panel S3	
	P1 before	P1 after	P3 before	P3 after	S3 before	S3 after
Bio-Rad HPLC	1.12	1.23	3.69	3.99	4.22	4.58
Siemens N latex CDT immunoassay	1.85	1.34	4.09	3.80	3.87	3.60
Sebia capillars CE	0.66	1.31	3.46	3.70	3.42	3.66
HPLC candidate reference	1.16	1.16	3.83	3.83	4.27	4.27
Mean	1.20	1.26	3.77	3.83	3.94	4.03
Intermethod CV	40%	6.4%	7.0%	3.1%	10%	12%

Table 2 Harmonization potential of candidate reference materials applied to mean %CDT (N Latex CDT immunoassay) or %disialotransferrin (HPLC and CE assays) results for three different samples obtained in EQA laboratories.

Results are shown before harmonization and after harmonization by the introduction of the corrective equation.

the CLSI C53A guideline. The next and final step of the WG-CDT will be a full standardization of commercial CDT methods against a reference method, using certified reference materials.

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Conflict of interest statement

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