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IFCC approved HPLC reference measurement procedure for the alcohol consumption biomarker carbohydrate-deficient transferrin (CDT): Its validation and use☆

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ABSTRACT

Carbohydrate-deficient transferrin (CDT) is used as a biomarker of sustained high alcohol consumption. The currently available measurement procedures for CDT are based on various analytical techniques (HPLC, capillary electrophoresis, nephelometry), some differing in the definition of the analyte and using different reference intervals and cut-off values. The Working Group on Standardization of CDT (WG-CDT), initiated by the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC), has validated an HPLC candidate reference measurement procedure (cRMP) for CDT (% disialotransferrin to total transferrin based on peak areas), demonstrating that it is suitable as a reference measurement procedure (RMP) for CDT. Presented is a detailed description of the cRMP and its calibration. Practical aspects on how to treat genetic variant and so-called di-tri bridge samples are described. Results of method performance characteristics, as demanded by ISO 15189 and ISO 15193, are given, as well as the reference interval and measurement uncertainty and how to deal with that in routine use. The correlation of the cRMP with commercial CDT procedures and the performance of the cRMP in a network of laboratories are also presented. The performance of the CDT cRMP in combination with previously developed commutable calibrators allows for standardization of the currently available commercial measurement procedures for CDT. The cRMP has recently been approved by the IFCC and will be from now on be known as the IFCC-RMP for CDT, while CDT results standardized according to this RMP should be indicated as CDT_{IFCC}.

1. Introduction and scope

1.1. General background

Transferrin is a glycoprotein synthesized in the hepatocytes that is present in serum at a concentration of ~2.0–3.5 g/L [1]. Transferrin is the major Fe^{3+} transporter and consists of a single polypeptide chain of 679 amino acids and has two binding sites for iron and two N-linked oligosaccharide chains attached to Asn432 and Asn630 [1]. At least 38 genetic transferrin variants due to amino acid substitutions have been reported [2]. Transferrin also occurs in different glycoforms due to variation in the oligosaccharide chains that can be bi-, tri-, and tetra-antennary. Each antenna is usually terminated by a sialic acid residue, and the total number of sialic acids forms the basis for naming of the glycoforms [3]. The transferrin glycoforms that are usually measurable in human serum are tetrasialotransferrin (~80%), pentasialotransferrin (~14%), trisialotransferrin (~4%), disialotransferrin (<2%), and hexasialotransferrin (~1%) [4–6].

In the mid-1970s [7], the amount of disialotransferrin was shown to increase in response to chronic heavy alcohol consumption and decrease again on abstinence with a half-life of about 10 days. The elevated value, which was accompanied by an increase in asialotransferrin, was

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termed "carbohydrate-deficient transferrin" (CDT) and suggested as a specific biomarker for chronic heavy drinking [8]. A large number of studies have since reported on the utility of CDT as a more specific alcohol biomarker [9–11] compared with traditional tests such as MCV, γ-GT, AST, and ALT. CDT levels related to various drinking levels, and identification of a few factors other than alcohol that may also elevate disialotransferrin, have been documented. Today CDT is considered an important objective indicator of sustained high alcohol consumption and is used to support the diagnosis of alcohol abuse and dependence in medical and forensic settings [12–15], including driver's license withdrawal and reinstatement.

Different measurement procedures have been used for quantification of CDT, including assays based on isoelectric focusing followed by immunofixation [16], anion-exchange column chromatography followed by turbidimetry [17], anion-exchange HPLC with measurement of the iron-transferrin absorbance maximum at ~470 nm [18,19], latex particle-enhanced nephelometric immunoassay [20], and capillary electrophoresis measuring the absorbance of the peptide bond at ~200 nm [21,22]. The transferrin glycoforms covered (i.e. the "CDT" analyte), and the way results are reported as either absolute or relative amounts, also differ between methods. Expressing CDT as percentage of total transferrin [19] was demonstrated to compensate for changes due to elevated (e.g. in iron deficiency) or lowered (e.g. in liver deficiency, iron overload, and inflammatory diseases) serum transferrin concentrations, and is hence the current standard. Despite this, CDT results obtained by different methods may differ significantly from each other, making comparison with previous results or reference ranges error prone. Obviously, physicians, forensic experts, lawyers, and patients prefer directly comparable results, irrespective of the method used.

This inconsistency prompted the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) to form a working group on standardization of CDT measurement (WG-CDT). The CDT standardization work started in 2005 and aimed to define the analyte, select and validate a reference measurement procedure (RMP) and reference materials, and propose a reference interval using the RMP. In the first WG-CDT publication [23], disialotransferrin (i.e. transferrin with one bi-antennary glycan chain [24]) was recommended as the single analyte on which the standardization is based, and the primary but not sole target for CDT measurement. It was further recommended that a well-defined qualitative and quantitative HPLC method for transferrin glycoforms [19] was suitable as a candidate RMP (cRMP), and that test results should be expressed as the relative concentration to total transferrin (% CDT). In a second publication [25], the HPLC cRMP was demonstrated to produce reproducible results within a reference laboratory network, and a candidate reference material was found commutable and stable on storage. These findings were the basis for further development of the cRMP which should be suitable for certification of secondary calibrators and control materials. In two subsequent WG-CDT publications [26,27], harmonization of currently employed CDT field methods was demonstrated possible and further established, using the "toolbox" of the International Consortium for Harmonization of Clinical Laboratory Results [28].

Laboratory standards (e.g. ISO 15193 [29]) demand detailed information about the analytical procedure and performance characteristics of a RMP. In this document, the HPLC cRMP for CDT [19] was further validated and demonstrated to fulfill the requirements of an IFCC RMP.

1.2. Scope of the HPLC candidate RMP

The HPLC cRMP is designed to quantify CDT for clinical or forensic purposes in samples of human serum. Analytical interference may occur in the presence of genetic transferrin variants [30] (see Section 5.3), but no interference of drugs therapies has been documented [31]. It has also been reported that severe liver disease can obscure the measurement and interpretation of analytical results [32,33]. The HPLC method is an optimised procedure and should be followed in every detail. The intended measurement interval is ~0.5%–16% disialotransferrin.

2. Basis of CDT standardization

2.1. Definitions and terms

The HPLC cRMP is intended to quantify the relative concentration of disialotransferrin in human serum, expressed as percentage of total transferrin, and calculated as the ratio of HPLC peak areas. Based on the IUPAC/IFCC format for designation of quantities in laboratory medicine [34], the WG-CDT proposes to define transferrin in serum as the system, and disialotransferrin as the component (analyte or substance of interest). The measurand is disialotransferrin measured by absorbance measurement of its iron-saturated glycan complex at 470 nm [35] after separation of glycoforms by anion-exchange HPLC. The kind of property is "substance fraction", as disialotransferrin is not an independent molecule but a % fraction (the unit) of total serum transferrin. The full measurand is called "P–Disialotransferrin/Transferrin; subst. fraction", according to IFCC C-NPU recommendations.

To distinguish between standardized and non-standardized CDT analytical procedures, values produced using standardized procedures (i.e. calibrated against the HPLC cRMP using secondary calibrators approved by the IFCC WG-CDT) are to be termed CDT_{IFCC}.

2.2. The standardization process

Mass spectrometry (MS) has been used for characterization of transferrin glycoforms purified from human albumin and immunoglobulin-free serum by salt precipitation followed by affinity and ion-exchange chromatography [24]. This also provided information on the structure of transferrin glycoforms. An MS method for transferrin glycoform glycopeptides obtained by tryptic digestion has been suggested as an alternative to routine procedures for CDT measurement [36]. However, because production and quantification of each transferrin glycoform with the needed purity is not yet solved, HPLC was proposed as the higher order available RMP [23]. The HPLC cRMP for CDT [19] fulfils the role as a RMP for certification of secondary calibrators and control materials, allowing for recalibration of all current CDT field methods to yield identical CDT_{IFCC} results (see Section 7.4.). According to recent definitions [37], the combination of a cRMP and certified standards fulfils the demands for method standardization.

3. Principle, apparatus, chemicals and reagents

3.1. Principle of the HPLC candidate RMP

The HPLC method is based on separation of the transferrin glycoforms by anion-exchange chromatography and NaCl gradient elution [19]. The global charge of each glycoform depends on the net charge of the amino acid chain [4], the number of iron molecules bound to the transferrin molecule, and the number of negatively charged terminal sialic acid residues on the glycan chains. The procedure is optimised for quantification of iron-saturated disialotransferrin in transferrin C homozygous subjects. Accordingly, the transferrin glycoforms differ in net charge only based on differences in sialic acid content.

3.2. Apparatus

The HPLC system should consist of a quaternary pump with a degasser for preparing the gradient, an autosampler suitable to inject 200 μL, a specified commercially available anion-exchange column, a high-sensitivity UV–vis detector, and a data management system allowing baseline peak integration with manual check. No special safety precautions are needed, other than usual for this type of equipment.

A typical set of equipment is obtained from Shimadzu (Kyoto, Japan) containing an LC-10ADvp pump equipped with a low pressure gradient control valve FCV-10ALvp (mixing chamber 550 μL), a DGU-14A degasser, and SIL-20AC autosampler. Absorbance detection at 470 nm

is performed with the UV–vis detector SPD-10AVvp. The UV–vis detector, which is critical, has the following specifications: cell path length 10 mm, band width 10 nm, wave-length accuracy ±1 nm, wave-length precision ±0.5 nm, drift 0.1 mAU/h, and noise level 5–10 μAU. Other HPLC systems which have been found suitable are, for example, Agilent 1100 and 1200, and Dionex UltiMate 3000.

Separation of transferrin glycoforms is performed using a Source® 15Q PE 4.6/100 anion-exchange chromatography column (GE Healthcare, Uppsala, Sweden) at room temperature (typically 20–25 °C).

3.3. Chemicals

All chemicals are of analytical grade, and the water is of HPLC grade (>18 MΩ). Chemical Abstract Service Registry Number (CAS), hazard class, H-, R- and S-phrases are reported where known.

1. Dextran sulphate sodium salt, Mw > 500.000, CAS 9011-18-1, harmful, R36-37-38, S 20-25-26-37-39 Sigma.
2. Nitrilotriacetic acid trisodium salt monohydrate (NTA) ($C_6H_6NNa_3O_6 \cdot H_2O$), Mw = 275.1, CAS 18662-53-8, harmful, neither R- nor S-phrases.
3. 2-[Bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol (Bis-Tris) ($C_8H_{19}NO_5$), Mw = 209.24, CAS 6975-37-0, harmful, neither R- nor S-phrases.
4. Calcium chloride dihydrate ($CaCl_2 \cdot 2\ H_2O$), Mw = 147.01, CAS 10035-04-8, harmful, H319, R36.
5. Iron(III) chloride hexahydrate ($FeCl_3 \cdot 6\ H_2O$), Mw = 270.3, CAS 10025-77-1, harmful, H302-315-318, R22-38-41.
6. Sodium hydroxide (NaOH), Mw = 40, CAS 12010-73-2, harmful, H314-318, R35.
7. Hydrochloric acid (HCl), Mw = 36.46, CAS 7647-01-0, harmful, H290-314-335, R34-37.
8. Sodium chloride (NaCl), Mw = 58.44, CAS 7647-14-5, non harmful.

3.4. Reagents and HPLC mobile phases

For separation of the transferrin glycoforms, column equilibration, and column cleaning, the following solutions are used [19]:

Mobile phase 1 (MP1) consists of 10 mmol/L Bis-Tris adjusted to pH 7.0 with 2 mol/L HCl.

Mobile phase 2 (MP2) is 10 mmol/L Bis-Tris containing 200 mmol/L NaCl adjusted to pH 6.2 with 2 mol/L HCl.

Mobile phase 3 (MP3) is 10 mmol/L Bis-Tris adjusted at pH 6.2 with 2 mol/L HCl.

Mobile phase 4 (MP4) is a cleaning solution consisting of 2 mol/L NaCl.

After preparation, all mobile phases are filtered through a 0.45-μm filter and degassed. The mobile phases are stable at 4 °C for 3 months.

Serum transferrin is iron-saturated using a FeNTA solution [19], prepared by dissolving 275 mg NTA and 270 mg $FeCl_3$ in 90 mL water. The pH is adjusted to 7.0 with 1.0 mol/L NaOH, and water is added to a final volume of 100 mL. The FeNTA solution is stored at 4 °C and is stable for 1 year.

The delipidation mixture is prepared by mixing equal volumes of 20 g/L dextran sulphate and 1.0 mol/L $CaCl_2$. The solution is stored at 4 °C and is stable for at least 1.5 months [19].

4. Sample

4.1. Sample type

The HPLC method is intended for measurement of transferrin glycoforms in human serum samples. Heparin plasma also seems suitable [19, unpublished results] but would need additional validation. EDTA plasma is not suitable, due to the existence of an additional asymmetric peak with a retention time similar to that of asialotransferrin, and citrate plasma causes a shoulder on the tetrasialotransferrin peak [19]. Bilirubin and haemoglobin at respective maximum concentrations of 150 μmol/L and 0.7 g/L do not cause marked interferences [19]. Hyperlipemia is not a source of potential interference, as the sample pretreatment includes delipidation.

Serum samples are obtained by standard phlebotomy and standard centrifugation procedure (e.g. 1500 g for 10 min). No special patient preparation is needed.

4.2. Sample preparation

Serum sample preparation includes complete iron saturation of transferrin using FeNTA, delipidation with dextran sulphate/$CaCl_2$, and centrifugation. Iron saturation is obtained by mixing 100 μL serum with 20 μL FeNTA solution. Subsequently lipoproteins are precipitated by adding 20 μL delipidation mixture. The sample is then gently mixed and stored at 4 °C for at least 4 h, or overnight. After centrifugation (3500 g for 5 min), 100 μL of the clear supernatant is diluted with 400 μL water and transferred to HPLC sample vials.

4.3. Sample stability

In centrifuged serum samples, the CDT level (disialotransferrin to total transferrin level in %) was stable for at least 5 days at room temperature [38]. At 4 °C, no significant change was observed after 10 weeks [36] or 3 months [27]. At −20 °C and −70 °C (Table 1), the stability of frozen samples was 24 and 36 months, respectively [27], and probably even much longer [39].

5. Details of the procedure

5.1. The HPLC method

5.1.1. Description of the HPLC method and data processing

Separation of transferrin glycoforms is performed on a Source 15Q PE 4.6/100 anion-exchange chromatography column at room temperature (20–25 °C), using linear NaCl gradient elution in Bis-Tris buffer at a flow rate of 1.0 mL/min. A 200-μL sample is injected on the column and the column is rinsed after separation with 2 mol/L NaCl to eliminate any protein residues. After reconditioning with injection buffer, the system is ready for another sample injection. Detection and quantification of the transferrin glycoforms is based on the absorbance maximum of the iron-transferrin complex at 470 nm [35]. Typical chromatograms for a low and a chronic heavy alcohol user are shown in Fig. 1.

HPLC peak integration and data processing are performed according to standard routines. The amount of each transferrin glycoform is quantified separately by peak integration using a baseline mode from the start of the disialotransferrin peak, or monosialotransferrin if visible, to the end of the hexasialotransferrin peak [19]. The transferrin glycoforms with more than six sialic acid residues (heptasialo- and octasialotransferrin) are present at very low concentrations, so the summed peak area for all glycoforms from asialo- to hexasialotransferrin is directly proportional to the total transferrin concentration. When detectable, asialotransferrin is integrated as an isolated fraction (Fig. 1B). This calculation method implies that the molar absorptivity of all iron-saturated glycoforms should be the same (see Section 5.2). The disialotransferrin (CDT) result is expressed as % of total transferrin. According to the NEN 1047 norm and

Table 1
CDT (% disialotransferrin) values by the HPLC cRMP after prolonged storage of a sample at two different temperatures (* $p < 0.05$ vs $time_0$).

Storage temperature (°C)	Storage time (months)					
	0	3	6	15	24	36
−20 °C	2,50	2,47	2,38	2,42	2,40	2,22*
−70 °C	2,50	2,46	2,46	2,44	2,40	2,44

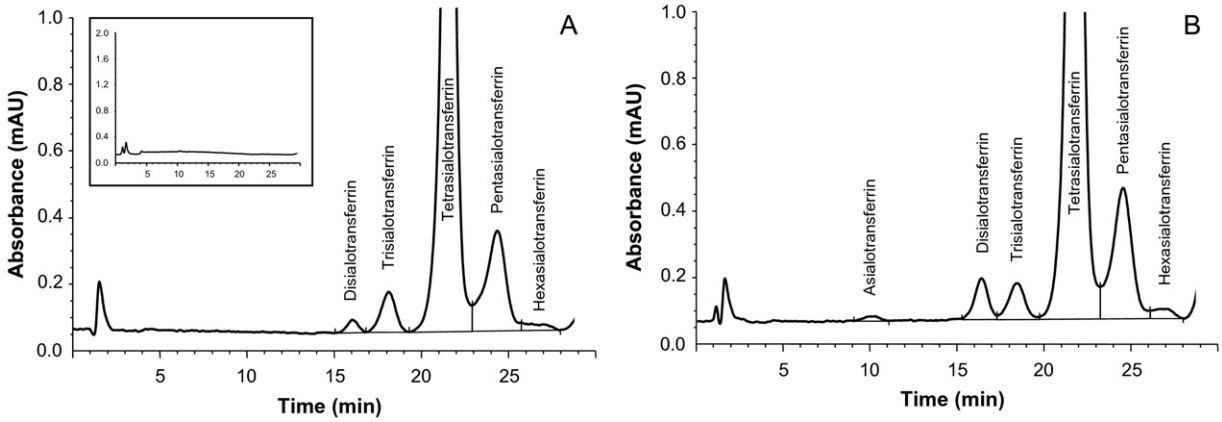

Fig. 1. HPLC chromatograms of serum from a healthy, light drinking control subject (A), the same sample after immunosubtraction of transferrin (A, insert), and from a chronic, heavy alcohol user (B) (data from [19]).

the European Committee for Standardization, the rounding should be 0.01%.

5.1.2. Structure of analytical series

In order to verify the performance of the HPLC equipment, control samples should be analyzed at the beginning and end of each analytical series. A matrix-free ready-to-inject test solution (e.g. ClinTest® CDT test solution from Recipe, Munich, Germany) allows checking the retention time of all glycoforms, peak shapes, and concentrations, without a possible bias due to improper sample preparation. Analytical or reagent blank samples are not needed.

The analysis can start, when all glycoforms are correctly separated, elute at the expected retention times, and peak shapes (or calculated number of plates) are satisfactory. Using the described sequence and gradient of mobile phases ensures no carry-over between samples.

For routine determination of % disialotransferrin by the HPLC cRMP, no replicate analysis is needed due to the low imprecision; the effect of single measurement on the imprecision is taken into account in the item devoted to measurement uncertainty. For measurement of reference materials, using three replicates is advised.

5.1.3. Quality control

At least one control sample is required to verify the performance of disialotransferrin concentration measurement. However, a two-level set of internal quality control samples is recommended, of which one with a disialotransferrin concentration near the upper limit of the reference interval. CDT controls are commercially available products or home-made serum pools stored at −20 °C or below. Participation in an external quality assurance scheme (EQAS) is mandatory, according to ISO 15189 [40].

5.2. Calibration of the HPLC method by gravimetry

Using primary calibrators: Isolated disialo- and trisialotransferrin and a mixture of tetrasialo- and pentasialotransferrin [24] were obtained from Bio-Rad Laboratories GmbH (Munich, Germany). A set of primary calibrators was prepared using transferrin-free serum (obtained by immunosubtraction [19]) spiked with the isolated glycoforms up to a total transferrin concentration of 3.0 g/L. These primary calibrators contained a fixed amount of trisialotransferrin (3–4% of total transferrin) and six equidistant concentrations (0–10%) of disialotransferrin. The mixtures were prepared by gravimetry to increase precision.

Two batches of these six primary calibrators were analyzed by the HPLC cRMP in two independent laboratories, using different lot numbers of the anion-exchange column and buffer batches (Fig. 2). A linear relationship between the target (gravimetry) and measured (HPLC) disialotransferrin concentrations was observed, the regression equations showing an almost perfect agreement between expected and measured concentrations (batch 1: $y = 1.0049x–0.0507$, $r^2 = 0.9998$; batch 2: $y = 0.9982x–0.0454$, $r^2 = 0.9996$). The slope of the regression equation was not significantly different from 1.00, and the intercept was not significantly different from the origin. This proves the suitability of using a relative concentration (% CDT).

Using secondary calibrators: Purified transferrin glycoforms obtained by preparative chromatography were assessed by the HPLC cRMP and MS analysis [24]. Experimental secondary calibrators were prepared by spiking disialotransferrin at five levels to a human serum pool with a disialotransferrin level of 1.1%, resulting in final concentrations of 1.1–9.0% disialotransferrin. The HPLC analysis of disialotransferrin of these spiked sera (Fig. 3) showed a high degree of correlation with the expected (calculated) values ($y = 0.941x–0.19$, $r^2 = 0.999$).

These two experiments provide indirect yet very strong evidence that the different iron-saturated transferrin glycoforms have the same molar absorption coefficients. Therefore, the relative peak area of each fraction corresponds to the relative amount of this glycoform to total transferrin and indicates that the HPLC cRMP does not require primary calibrators. Peak areas of iron-saturated serum transferrin glycoforms can be used as the measurand. Consequently, target values of secondary calibrators derived from peak area ratios can be assigned, by analysis in the reference laboratory network using the cRMP.

5.3. Limitations of the HPLC method – special cases

High levels of bilirubin (>150 μmol/L) and haemolysis (Hb > 0.7 g/L), alone or in combination with a low serum transferrin concentration, can cause severe HPLC baseline sloping and loss of accuracy of peak area integration for the minor transferrin glycoforms. Difficulties in the separation of glycoforms, and consequently in peak integration, may occur in genetic transferrin variant samples, and in cases of acquired changes in transferrin such as severe liver disease. Elevated CDT levels may occur in rare cases of inherited metabolic diseases known as congenital disorders of glycosylation (CDG), and in fructosemia and galactosemia [41], whereas common disorders (e.g. diabetes) [42] or medications (e.g. antiepileptic drugs) [31] do not interfere.

5.3.1. Genetic variants of transferrin

In Caucasians, transferrin-C is the wild type allele with a prevalence of ~95%. The subtypes C1 (wt) and C2 (Pro570Ser) have allele frequencies of about 75 and 19%, respectively [43], resulting in a frequency of about 59% for the C1-1 and 23% for the C2-1 phenotypes as the main forms. Heterozygous transferrin-BC and transferrin-CD forms are present in ~1% of Caucasian populations [30,44]; these heterozygotes are, however, more common in certain Asian [45,46], African, and South American populations [44].

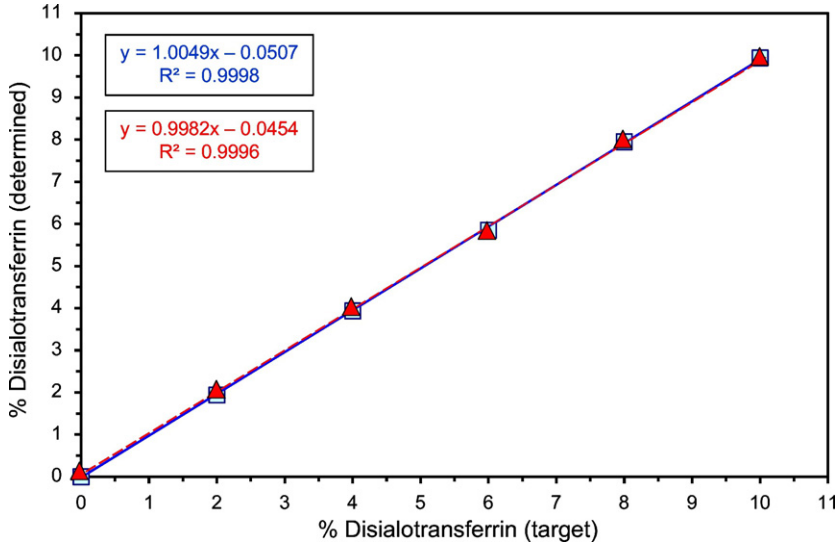

Fig. 2. Measurement of CDT (% disialotransferrin) by the HPLC cRMP in two batches of primary calibrators at six levels of disialotransferrin in the same mixture of purified pentasialo-, tetrasialo- and trisialotransferrin prepared by weighing (by courtesy of Bio-Rad Laboratories GmbH, Munich, Germany).

The HPLC cRMP is optimised for measurement of homozygous transferrin-C subjects. Heterozygous variants like transferrin-BC and transferrin-CD present "abnormal" HPLC profiles with a superimposed double pattern of glycoforms [30], resulting respectively from transferrin B (elutes after C in the HPLC method) and C, or transferrin C and D (elutes before C) glycoform chromatographic separation. Exact quantification of "total" disialotransferrin is not possible for transferrin-BC or transferrin-CD variants, due to overlay of the individual allele peak patterns. Generally the presence of a genetic variant is easily recognized by a double peak of the major tetrasialotransferrin form (e.g. one originating from the C allele and one from the B or D allele). Transferrin-C subtypes, C1, C2, and most C1-C2 are suitable for quantification with the HPLC cRMP. However, for a recently described C2 subtype [47], HPLC proved to be not suitable. Transferrin mutations can affect also the oligosaccharide-chain binding sites at Asn432 and Asn 630. Thus, a mutation at one of these sites abolishes N-glycosylation and the mutated transferrin molecule can bear only one oligosaccharide chain, which increases the proportion of disialotransferrin. This has been described both for Asn432 [48,49] and Asn630 [49,50].

5.3.2. High trisialotransferrin and "di-tri bridging"

Some serum specimens show an elevated amount (>6%) of trisialotransferrin. The concentration of this glycoform is not dependent on alcohol intake and the increase is typically accompanied by a measurable quantity of monosialotransferrin [51].

In other samples, baseline separation between disialo- and trisialotransferrin is not achieved, due to the presence of transferrin fractions eluting in-between the two (Fig. 4). This so-called "di-tri bridging" phenomenon is not caused by the presence of a genetic variant and has been described for C1-C1, C1-C2, and C1-C3 allele combinations [32]. The di-tri bridging is typically associated with severe liver disease (e.g. cirrhosis) [32,33], and often occurs together with an elevated trisialotransferrin level. The di-tri bridging hampers accurate quantification of disialotransferrin by the HPLC cRMP, and is due to increased branching and fucosylation of the oligosaccharide chains [52].

5.3.3. CDG syndrome

In CDG, mutations in the genes coding for enzymes involved in the synthesis of glycans (oligosaccharide chains) lead to a variety of

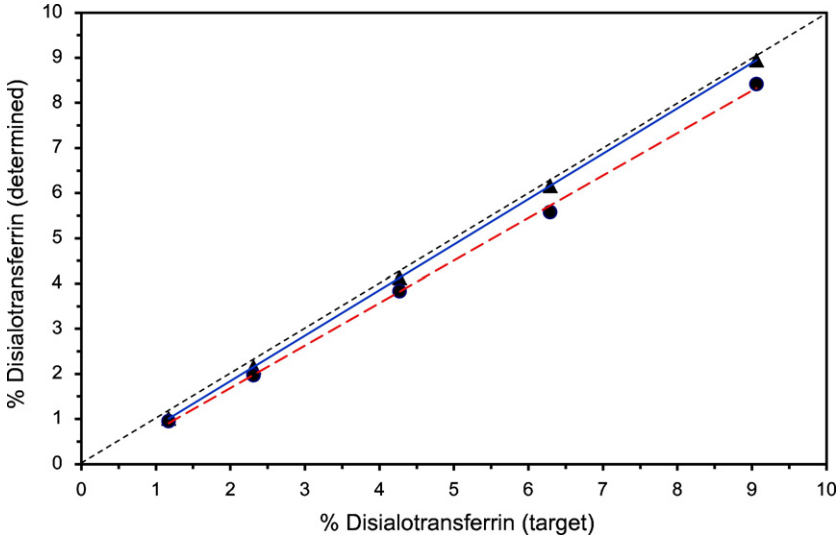

Fig. 3. Measurement of CDT (% disialotransferrin) by the HPLC cRMP in a five-level secondary CDT reference material (determined; y axis) prepared by spiking a low % disialotransferrin serum with weighted amounts of purified disialotransferrin (data from [24]), compared to the target values (x axis).

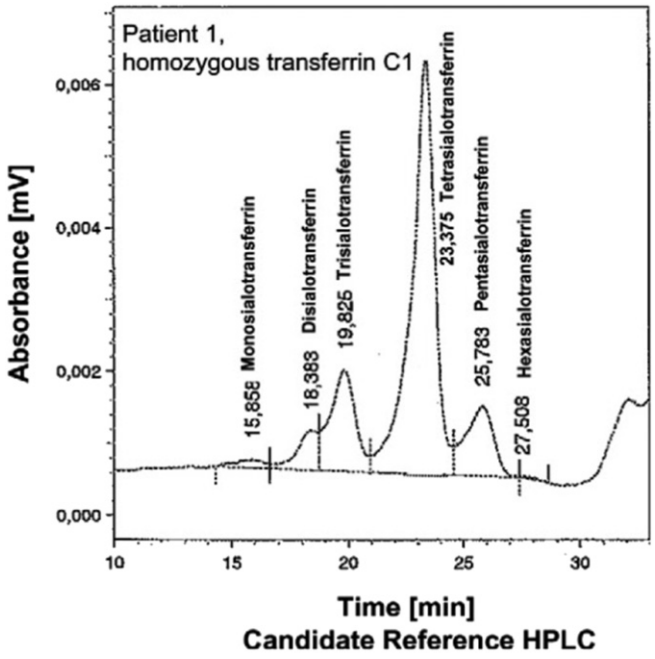

Fig. 4. Transferrin glycoform pattern in serum of a cirrhotic patient showing a loss of chromatographic resolution of disialotransferrin from trisialotransferrin ("di/tri bridging") due to fucosylation of transferrin. Genotyping showed a usual homozygous C1 type (data from [32]).

abnormal glycosylation patterns of transferrin, depending on the enzyme affected. The transferrin glycoform profiles obtained in HPLC analysis are typical of these diseases, often leading to highly elevated disialotransferrin values (>20%) [53], and may be used for preliminary screening of CDG into Type-I or Type-II. It should be noted that CDG testing and diagnosis is usually done in newborns, because many CDG patients suffer from mental and psychomotor retardation that are evident already from an early age.

6. Validation of the HPLC candidate RMP

6.1. Detection limit

Low end-precision performance characteristics at the detection limit are very important if the result may have clinical or forensic significance, which does not apply in the case of very low CDT results. Medical or forensic CDT requests are typically used in case of suspected chronic heavy alcohol use, or for routine company health care testing [54], and are especially meaningful when the CDT level exceeds the upper level of the reference interval of 0.6–1.7% (see Section 8).

The limit of detection (LOD) and the lower limit of quantification (LLOQ) of the method should be well below the upper level, and preferably also below the lower level, of the reference interval, to ensure reliability of elevated results. According to the EP17-A2, the LOD = mean of blanks + 1.645 SD_{blank} + 1.645 $SD_{low\ sample}$ leading to a calculated LOD of 0.16% disialotransferrin, which is close to the empirically [19] estimated value of 0.10%. For the LLOQ, it has been proposed to use 10 $SD_{low\ sample}$ which means an LLOQ of 0.8% disialotransferrin, but the factor 10 is arbitrary. We instead prefer to use 5 $SD_{low\ sample}$ which means an LLOQ of 0.4% disialotransferrin. Another approach is a calculation of the LLOQ based on imprecision. From the results presented in Section 6.3, it is estimated that the LLOQ at a CV of 10% is well below the level of 1.0% disialotransferrin.

It should be noted that these analytical thresholds depend on the transferrin concentration in the sample and the performance characteristics of the UV–vis detector. As very few disialotransferrin values below 0.5% are observed when running human sera, the analytical sensitivity of the HPLC cRMP is considered sufficient to avoid any clinical or forensic consequence related to the quantification of CDT.

6.2. Linearity

Linearity and serum transferrin concentration: CDT was measured in a serum sample containing a very high transferrin concentration (~5 g/L) at different dilution ratios (10–75% serum) with water [19], and the measured sum of peak areas were compared to the calculated value. The correlation was linear ($r^2 = 0.999$) and the relative amount of disialotransferrin unaffected by dilution. The HPLC cRMP is therefore considered linear over a transferrin concentration range of at least 0.5–5 g/L, thereby well covering the reference interval for serum transferrin (2.0–3.5 g/L). This enables use of the HPLC cRMP also in samples with an abnormally low transferrin concentration (<1.4 g/L).

In addition, using variable sample injection volumes in the range 50–250 μL did not affect the relative quantification of disialotransferrin (% CDT). Even a 10-μL injection volume has been used in CDG testing on newborns, where the serum sample volume may be limited [51].

Linearity and disialotransferrin content: The linearity of the HPLC cRMP was assessed by measuring CDT in three sets of serum samples. Each set was constituted by mixing (from 9:1 to 1:9, v/v) a high CDT sample (16.30%, 16.28%, and 15.43% disialotransferrin, respectively) with a low CDT sample (1.02%, 1.02%, and 1.07%, respectively). The correlation between calculated and measured values was high ($r^2 = 0.999$), and the regression equation ($CDT_{[measured]} = CDT_{[calculated]} \times 0.997$ [95%CI 0.985–1.010] + 0.017 [95%CI −0.096–0.131]) (Fig. 5) showed the equivalence between expected and measured concentrations.

6.3. Imprecision

The reproducibility of the HPLC cRMP was assessed by repeated measurements of pooled serum samples, according to the CLSI EP05-A2 procedure [55]. Two pools (low and high CDT) were prepared and analyzed in duplicate in two separate runs over 20 days. No outlier was detected during this experiment. As indicated in Table 2, the within laboratory imprecision was <5%, which is in accordance with previous results [19]. This result was confirmed in another experiment by repeated measurement (n = 34) on non-consecutive days of two sets of pooled sera (a low CDT pool with 1.20%, and a high CDT pool with 3.07% disialotransferrin), yielding coefficients of variation (CV) of 7.0% and 2.9%, respectively.

The inter-laboratory imprecision has been quantified in a laboratory network running the HPLC cRMP, both with frozen and lyophilized samples. Six ring trials have been conducted from 2004 to 2013 and, as expected, the imprecision decreased when the disialotransferrin concentration in the sample increased. The inter-laboratory CV ranged from 5.6% for low CDT samples to 3.7% for high CDT (elevated) values [25,27]. These data were compared to the results from an EQAS in 2013–2015 (Equalis, Sweden). Based on 28 test results from 15 to 20 laboratories using single measurement of fresh serum samples, the mean inter-laboratory CV was 5.8% at a mean CDT level of 2.3%.

6.4. Measurement uncertainty

Both the ISO 15189 and ISO 15193 require determination of measurement uncertainty, when reporting an analytical result. The measurement uncertainty concept is defined in the "Guide to the expression of uncertainty measurement" (GUM) [56] and is briefly summarized below. In this model, the composite uncertainty (u) can be calculated [57], using IQC, EQC, and intra-individual biological variation data by the following equation:

$$u_{composite} = \sqrt{(u^2_{IQC} + u^2_{EQC} + u^2_{intra\text{-}individual})}$$

where $u^2_{(IQC)}$ is the variance of internal quality control, $u^2_{(EQC)}$ the variance related to accuracy and dispersion of external quality control

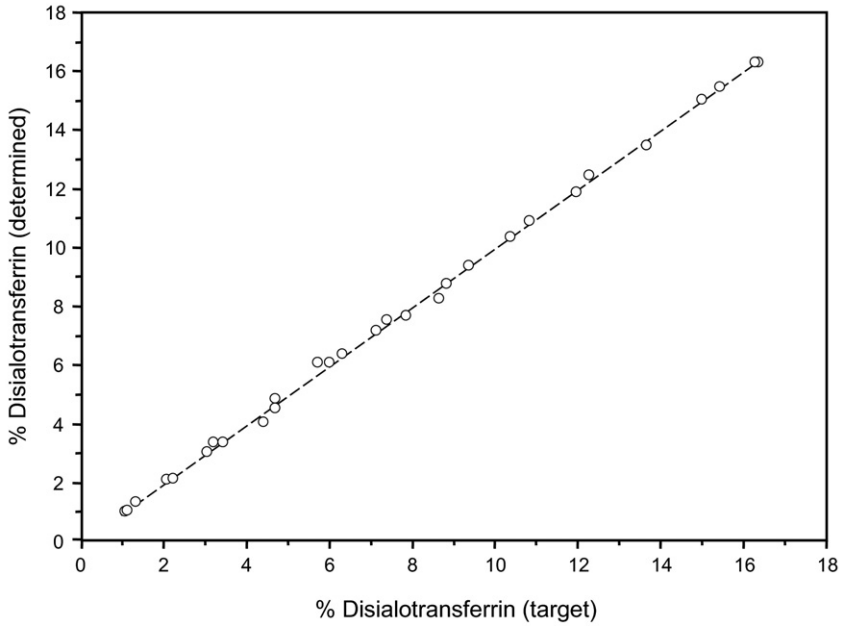

Fig. 5. Measurement of CDT (% disialotransferrin) using the HPLC cRMP in serial dilutions of high CDT samples with low CDT samples (determined; y axis), compared to the calculated values (target; x axis) (Pearson's correlation coefficient r = 0.999).

values, and $u^2_{(intra\text{-}individual)}$ the intra-individual variance. The unit of u is the same as for the measured analyte [57], hence % in case of CDT measurement. The $u^2_{(IQC)}$ value was calculated from the repeated CDT pool measurements and found to be 0.0076% (considered as the intra-laboratory variance). The $u^2_{(EQC)}$ value was calculated from the 28 EQAS results mentioned above in Section 6.3 (CDT from 1.96% to 3.75%, mean 2.17%) and was 0.0097% (considered as the inter-laboratory variance).

For calculating the $u^2_{(intra\text{-}individual)}$ variance, 17 subjects with repeated CDT measurements (n = 2–4) and at least one month between two consecutive measurements were used. Patients were in "alcohol steady state" conditions during the study period and had CDT values in the ~1–2% range (overall mean value 1.72%). The CV of each set of two consecutive measurements for each patient was calculated, showing a mean CV of 6.5%. This result was considered as the intra-individual variation and allowed the calculation of $u^2_{(intra\text{-}individual)}$ found at 0.0070%. The resulting value of the composite uncertainty $u_{(composite)}$ was then calculated and found to be 0.156%.

According to the NIST guideline 1297 [58], "what is often required is a measure of uncertainty that defines an interval about the measurement result y within which the value of the measurand Y is confidently believed to lie". The measure of uncertainty intended to meet this requirement is termed expanded uncertainty (U). The expanded uncertainty is obtained by multiplying the composite uncertainty with a coverage factor k [58]. A 95% confidence interval (CI) corresponds to a k-value of 1.96, and a 99% CI to a k-value of 2.58.

The ISO 15193 advocates reporting clinical results with a measurement uncertainty. For forensic purposes, a CI of either 95% or 99% is required [59,60], corresponding to a coverage factor of 2 or 3. The expanded uncertainty for CDT measurement with the HPLC cRMP was calculated, using a k-value of 2 (95% CI), resulting in a U value of 0.312% at a CDT concentration around 1.7%.

Another component of uncertainty is the accuracy of the measurement, defined by a bias (E) to the "true" value. In a given laboratory using a given procedure, the true value is expressed as follows: $CDT_{(true)} = CDT_{(measured)} + E \pm U$ at 95% CI. The HPLC cRMP has been developed as a standardization procedure and thus no bias factor E has to be applied. Therefore, a CDT result (in %) obtained can be expressed as follows: $CDT_{(true)} = CDT_{(measured)} \pm 0.31$ at 95% CI (see also Section 8).

6.5. Analytical specificity

After incubating serum samples with anti-transferrin antiserum (i.e. immunosubtraction), no peaks were observed in the HPLC chromatogram at 470 nm (see Fig. 1A, insert) [19]. This demonstrated the specificity of the absorbance measurement of transferrin glycoforms by the HPLC cRMP in serum samples (see Section 4.1).

7. Method comparison and standardization

7.1. Capillary electrophoresis methods

Three capillary electrophoresis (CE) procedures for CDT have been compared with the HPLC cRMP, following the manufacturer's instructions for use. The results produced by the CEofix™ kit (Analis, Suarlee, Belgium) [61] (CE_1) run on a mono-capillary electrophoresis system were highly correlated ($r^2 = 0.972$) with those obtained by the HPLC cRMP. The Capillarys (Sebia, Evry, France) multi-capillary method

Table 2
Repeatability, day-to-day, and within-laboratory imprecision of CDT (% disialotransferrin) measurement with the HPLC cRMP at two different levels, according to the CLSI EP05-A2 protocol.

Sample	Mean CDT value (% disialotransferrin)	Repeatability		Between day		Within laboratory	
		SD	Imprecision	SD	Imprecision	SD	Imprecision
Low pool	1,18%	0,046	3,90%	0,026	2,20%	0,053	4,50%
High pool	3,05%	0,045	1,50%	0,028	0,90%	0,053	1,80%

(CE_2) results were also highly correlated with the HPLC cRMP [56,57], although the CE_2 method produces consistently lower CDT results ($r^2 = 0.996$; $CE_2 = 1.074 \times$ HPLC – 0.46 [62], and $r = 0.986$; $CE_2 = 0.968 \times$ HPLC − 0.248 [63]). Also the recently introduced V8 CDT (Helena Biosciences, Tyne and Wear, UK) procedure (CE_3) produced consistently lower CDT results than the HPLC cRMP ($r^2 = 0.979$; $CE_3 = 0.854 \times$ HPLC − 0.03). Similar results ($r^2 = 0.941$; $CE_3 = 0.84 \times$ HPLC + 0.03) were obtained in another study [64].

7.2. HPLC methods

Two commercial HPLC assays for CDT have been evaluated and compared with the HPLC cRMP. The %CDT-by-HPLC method (Bio-Rad, Hercules, CA) [65] run on an Agilent system showed a high correlation with the HPLC cRMP but produced slightly lower results ($r^2 = 0.998$; %CDT-by-HPLC $= 0.987 \times$ cRMP − 0.205).

A study carried out to evaluate the correlation between the ClinRep® CDT (Recipe, Munich, Germany) and the cRMP HPLC procedures, using 77 serum samples covering a wide range of CDT concentrations (0.5–16.1%), showed good agreement between the methods ($r^2 = 0.993$; ClinRep CDT $= 1.017 \times$ cRMP [95% CI 0.997–1.037] − 0.007 [95% CI −0.196–0.064]) (Fig. 6). The Cusum test also indicated a linear agreement, and a paired *t*-test confirmed the absence of a significant difference between the results obtained by both HPLC methods, thereby confirming the results of a previous study [66].

7.3. Immunochemical method

The CDT results of a homogeneous latex-enhanced immuno inhibition nephelometric assay, based on a monoclonal antibody directed against CDT (N Latex CDT; Siemens, Marburg Germany) [20], were compared with those of the HPLC cRMP. Although the immunoassay is based on a different analytical principle than HPLC, the comparison showed good correlation but a systematic bias and slope ($r = 0.989$; N Latex CDT $= 0.838 \times$ cRMP CDT + 0.354). The N-Latex CDT immunoassay appears not to be influenced by the common transferrin variants [20].

7.4. Standardization procedure

The wide range of upper reference limit values for "CDT" given by the manufacturers of commercial assays (from 1.3% for Sebia [63] to 2.35% for Siemens [20]), and the variable regression equations given above, clearly demonstrated the urgent need for standardization of CDT measurement. The HPLC cRMP was shown to yield homogeneous and stable results over time in an international network of six reference laboratories, using frozen serum samples as control materials [25–27]. CDT was measured by the network laboratories in a five-level set of calibrators produced at the MCA Laboratory (Queen Beatrix Hospital, Winterwijk, The Netherlands) to assess target values. The manufacturers of the above mentioned commercial CDT procedures then measured CDT using their standard procedure. The inter-method CV was initially 8.8% but decreased to 3.4% after mathematical re-calibration.

A set of 66 serum samples (CDT range 1.10–6.10%) was analyzed by seven commercial CDT procedures and the HPLC cRMP, before and after standardization. The correlation and the concordance between different procedures were tested, using the Pearson's test. The concordance correlation coefficient ρ_c evaluates the degree to which pairs of observations fall on the 45° line through the origin [67]. It comprises a measurement of the coefficient of correlation ρ, which reflects the precision, and the calculation of a bias correction factor C_b, which is a measure of accuracy. Finally, the concordance correlation coefficient is calculated using the formula: $\rho_c = \rho \times C_b$.

A scale of interpretation of the concordance correlation coefficient values ρ_c was used, to estimate agreements between the HPLC cRMP and each field method. The strength of agreement was classified according to McBride [68] into four categories: "almost perfect" ($\rho_c > 0.99$), "substantial" ($0.95 > \rho_c > 0.99$), "moderate" ($0.90 > \rho_c > 0.95$) and "poor" ($\rho_c < 0.90$). In head-to-head comparison of the seven commercial procedures, only nine of 21 pairs were considered as "substantial" and one as "almost perfect", whereas, after standardization, the number of "substantial" pairs increased to 19 (Table 3). When comparing the commercial methods with the HPLC cRMP, the number of "moderate" agreements decreased from four to zero and the number of "almost perfect" increased from zero to three, whereas four pairs had a "substantial" agreement both before and after standardization. These data clearly

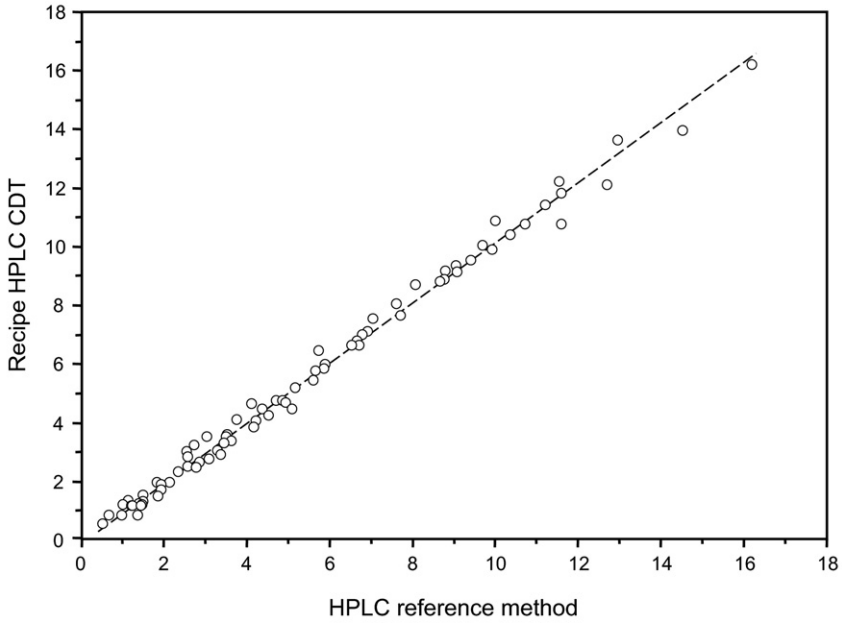

Fig. 6. Measurement of CDT (% disialotransferrin) values by the Recipe® Clin Rep HPLC method and the HPLC cRMP in 77 patient samples.

Table 3
Strength of agreement, according to McBride [68], in head-to-head comparison of seven commercial procedures themselves, and between these procedures and the HPLC cRMP, before and after standardization using the secondary calibrators.

Comparisons	Agreement categories	Strenght of agreement	
		Before standardization	After standardization
Between commercial procedures	Poor (<0.90)	5	0
	Moderate (0.90–0.95)	6	1
	Substantial (0.95–0.99)	9	19
	Almost perfect (>0.99)	1	1
Between commercial procedures and the RMP	Poor (<0.90)	0	0
	Moderate (0.90–0.95)	3	0
	Substantial (0.95–0.99)	4	4
	Almost perfect (>0.99)	0	3

demonstrated that standard materials calibrated by the HPLC cRMP can be used for standardization of the commercial CDT procedure results [27].

8. Reference interval

The 2.5–97.5 percentile reference interval for "social drinkers" was initially estimated to be 0.67–1.67% [19]. Later, a set of 245 samples from a collaborative WHO-ISBRA study on different populations [9], and 97 control subjects from the Karolinska University Hospital (Stockholm, Sweden), were used to calculate a reference interval according to CLSI C28-A3. In the 342 samples, the mean CDT value was 1.12% and the calculated central 95% reference interval plus CI was 0.71% disialotransferrin (90% CI 0.68–0.75) to 1.60% (90% CI 1.51–1.70%). No clinically significant sex difference in CDT was noted for non-drinkers [9,46]. However, pregnancy was demonstrated to increase CDT measured with the HPLC cRMP [69], yielding results in the high normal or slightly elevated range at the end of pregnancy (i.e. in the third trimester), and returning to normal within 2–3 months post-partum.

An independent way to obtain a reference interval is the use of Bhattacharya calculation [70]. This procedure allows the determination of reference values from unselected populations with an underlying Gaussian or Gamma distribution of "normal" values. In a set of 5000 patient CDT results obtained in Malmö (Sweden) with the HPLC cRMP, the reference interval was found to be 0.77–1.72% (central 95%) using a Gamma distribution, or 0.68–1.65% using a Gaussian distribution (Wielders and Naus, 2009, unpublished). Combining these data, it was concluded that the upper reference limit (URL) for the HPLC cRMP will be 1.7%, which agrees with that originally reported [19].

For forensic applications, the use of measurement uncertainty is recommended [59] and fulfils the recommendations of the ISO 15189. Using the expanded uncertainty calculated above at 95% CI, a true CDT value of 1.7% could be, at most, measured as 2.0% in a single measurement.

This 2.0% cut-off value guaranties a high level of specificity and could be considered in forensic medicine as the highest numerical value to be expected for CDT, using the HPLC cRMP in occasional or non-drinkers considering all sources of imprecision.

9. Conclusion and approval by the IFCC

The IFCC initiative for standardization of the alcohol biomarker CDT [71] is completed with the description and validation of a candidate reference measurement procedure, as presented in this work. The procedure and its use are described in detail, along the general guidelines of ISO 15189 and ISO 15193, fulfilling the demands for a RMP and allowing for standardization of the commercial CDT assays. As such, this measurement procedure is approved by the IFCC Working Group on CDT, by the Executive Committee of the Scientific Division of the IFCC and accepted by the IFCC National Societies. From now on it will be known as the IFCC-RMP for CDT, while CDT results standardized according to this RMP should be indicated as CDT_{IFCC}.

Conflict of interest

The authors declare that they have no competing financial interests.

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