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A prolonged time interval between blood sample collection and centrifugation causes an increase in serum carbohydrate-deficient transferrin

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Summary

Background: Carbohydrate-deficient transferrin (CDT) is used for the laboratory diagnosis of chronic alcohol abuse. Non-optimal preanalysis can cause an increase in CDT and false positive results. The aim of our study was to determine whether CDT results change over time between collection of the blood sample and centrifugation, and whether shipment of whole blood samples is a potential source of false positive CDT reports.

Material/Methods: 152 blood samples were drawn from 38 persons (4 tubes per person, one venipuncture) and randomly assigned to 4 groups with different time intervals between blood sample collection and centrifugation (1h, 24h, 48h, 144h). CDT analysis was done using the ChronAlcoI.D. assay. The statistical analysis was based on box-plots, ANOVA and Kruskal-Wallis ANOVA.

Results: The means and medians of CDT increased with the time of whole blood storage. ANOVA analysis of between-group differences was significant for mean CDT concentrations between 1 and 144 hours of whole blood storage. There was no correlation between CDT and free hemoglobin as a measure of hemolysis. An interference of hemolysis with CDT measurement can be excluded as the main cause of increased CDT results with whole blood storage time. Whether an in vitro degradation of the transferrin N-glycan chains causes the CDT increase should be evaluated by isoelectric focusing of the transferrin isoforms in a further study.

Conclusion: Storage or shipment of whole blood samples can shift initially normal CDT values to borderline and borderline to pathological CDT results.

key words: alcohol • blood • CDT • hemolysis • preanalysis

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BACKGROUND

Carbohydrate-deficient transferrin (CDT) is widely used for the laboratory diagnosis of chronic alcohol abuse. A review of the pathobiochemistry, preanalysis, analysis and postanalysis (interpretation) has been published recently [1]. In contrast to analysis and interpretation, the assessment of the preanalysis of CDT has been almost completely neglected. As a consequence, issues of CDT preanalysis which are important for reliable CDT measurement still remain to be investigated [1]. One important question is whether or not serum CDT is affected by the time that elapses between the collection of the blood sample and its centrifugation to separate the serum from the blood clot. This issue gains importance from the fact that clinical laboratory analysis is increasingly done in satellite laboratories, and that many medical facilities (even small clinics or alcohol abuse treatment centers) have no centrifuge. In these cases, the laboratory receives whole blood samples which have been drawn one day (most often) or several days earlier. Since in fact non-optimal serum storage can cause an increase in CDT and false positives regarding chronic alcohol abuse [1], the same problems can be expected with whole blood samples. False positive results can have serious social and employment consequences for the person tested, and should be reduced to a minimum.

The purpose of the present study, then, was to evaluate whether CDT results change with time in whole blood samples, and to determine whether the shipment of whole blood samples is a potential source of false positive CDT reports.

MATERIAL AND METHODS

All the participants in the study gave their informed consent. The study was carried out according to the provisions of the 1975 Declaration of Helsinki, as revised in 1996.

Blood was taken from 38 healthy male and female volunteers (medical students, staff of the laboratory) by one venipuncture. 4 blood tubes (a-d) were drawn from each person, resulting in a total of 152 blood samples numbered Ia, Ib, Ic, Id, IIa, IIb, IIc, etc. Four different time periods between blood sample collection and centrifugation were assessed, mimicking the normal and delayed postal shipment of the whole blood sample to the laboratory: 1h (immediately after clotting), 24h, 48h, and 144h. The blood samples were assigned to these four groups in a randomized manner, e.g. Ia in 1h, Ib in 144h, Ic in 24h, Id in 48h, IIa in 144h, IIb in 24h, IIc in 48h, IId in 1h, etc. Shipment of the blood samples was simulated by transportation in a car on the daily trip to and from the laboratory (about 2. 5 h per day, temperature inside the car 20–25°C). After the defined period (1h, 24h, 48h or 144h after blood drawing), the corresponding blood tubes were centrifuged at 2000g for 10 min, the serum separated from the blood clot and frozen at –22°C until analysis. All serum samples were thawed only once, on the day of analysis, and analysed in the same analysis run to avoid additional inter-assay variance.

CDT was determined by the ChronAlcoI.D. assay (Sangui BioTech Inc, Santa Ana, U.S.A. distributed by Biodiagnostics, Kiel, Germany) in accordance with the manufacturer's instructions. For CDT- and non-CDT fractionation, 100 µL of serum or control sample and 500 µL of metal^{3+}-ion reagent (for transferrin binding site saturation) were mixed and incubated for 5–10 min at room temperature. A 500 µL aliquot of this mixture was applied to a microcolumn, and the effluent with non-CDT isoforms was discarded. After a rinsing step, CDT isoforms were eluted and the eluate was used for the final turbidimetric immunoassay. Preparation of the total transferrin solution was done while microcolumn separation was taking place. 20 µL of metal^{3+}-ion saturated serum or control sample were mixed with 800 µL of elution buffer. This sample was used in the turbidimetric immunoassay to determine the total serum transferrin concentration. CDT and total transferrin were quantified by a microtiter-plate turbidimetric immunoassay. 200 µL of calibrators, microcolumn eluates and total transferrin dilution samples were pipetted directly to the bottom of each well, and atypical absorbency (background) was read at 405 nm (Dynatec MR 5000 reader, Dynex Technologies, Denkendorf, Germany). Following this, 100 µL of transferrin antibody solution was added to each well and the final absorbency was read after 15 min at room temperature.

The analysis data was analyzed by means of the Dynex Revelation 3.2 software program (Dynex Technologies, Denkendorf, Germany). The results were reported as CDT/transferrin ratios. The cut-off used as the decision criterion was 2.5–2.7% for the CDT/transferrin ratio [2].

Quality control was done in compliance with the recommendations published in the Guidelines of the German Federal Medical Association. The precision and accuracy of CDT measurement were checked by two control samples with normal and pathological CDT/transferrin ratios (CDT control set, Sangui BioTech Inc, U.S.A.) placed at the beginning and the end of the run. The CVs for the low and high controls were <7.5% and <7.9% in the appropriate quality control period. A detailed study on the intra- and inter-assay variance of the ChronAlcoI.D. assay is given in [2]. The laboratory has regularly participated successfully in external quality control programs.

Free hemoglobin was measured spectrophotometrically using a test kit supplied by Sigma Diagnostics (Deisenhofen, Germany). Quality control was done in accordance with the Guidelines of the German Federal Medical Association as described above.

Statistical analysis

Statistical analysis was done by using the Analyse-it for Microsoft Excel program (Analyse-it Software, Ltd, Leeds, United Kingdom). The mean (including 95%

Table 1. Impact of time elapsing between blood sample collection and centrifugation on serum CDT

CDT [%]		n	mean	SD	95% CI	median	95% CI	25th perc.	75th perc.
Whole blood storage	1h	38	2.3	0.5	2.2–2.5	2.3	2.1–2.5	1.9	2.6
	24h	38	2.4	0.5	2.2–2.6	2.4	2.2–2.7	2.0	2.7
	48h	38	2.5	0.6	2.4–2.7	2.6	2.4–2.8	2.2	2.9
	144h	38	2.6	0.5	2.5–2.8	2.6	2.4–2.7	2.4	3.0

Abbreviations: CI - confidence interval; SD - standard deviation; perc. - percentile

Table 2. Significance of serum CDT increase with whole blood storage time assessed by one-way between-subjects analysis of variance (ANOVA)

Source of variation	Sum of squares	Degrees of freedom	Mean square	Ratio mean square/mean square within groups	p
CDT [%]	2.27	3	0.76	2.81	0.042
Within groups	39.85	148	0.27		
Total	42.12	151			

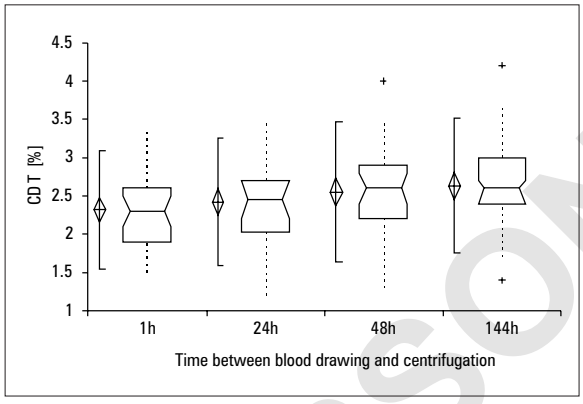

Figure 1. Box-plots for serum CDT depending on the time between blood sample collection and centrifugation. The left image of each time group shows the mean (center of the prism), the 95% confidence interval (upper and lower tips of the prism) and the parametric percentile range (vertical line). The right image of each time group shows the Box-plot with median (horizontal line within the box), 95% confidence interval of the median (indicated by the ends of the diagonal lines), interquartile range, corresponding to 25th to 75th percentile (lower and upper horizontal line of the box), nearest observations within 1.5 interquartile ranges (dotted line) and near outliers between 1.5 and 3.0 interquartile ranges distant (indicated by +).

confidence interval and standard deviation), median (including 25th and 75th percentile), frequency histogram (not shown), Shapiro-Wilk coefficient, skewness and kurtosis of the distribution of the CDT values were computed for each of the four groups (cf. 'Results' and Table 1). Box-plots were computed and arranged side by side in Fig. 1. The significance of the differences in the means and medians between the groups was tested by (parametric) one-way between-subjects ANOVA and (non-parametric) Kruskal-Wallis ANOVA (Table 2). The significance value was set at 95% or p = 0.05 throughout.

Contrast	difference	95% Confidence interval*
1h vs 24h	-0.097	-0.403 to 0.208
1h vs 48h	-0.232	-0.537 to 0.074
1h vs 144h	-0.318	-0.624 to -0.013 significant
24h vs 48h	-0.134	-0.440 to 0.171
24h vs 144h	-0.221	-0.527 to 0.085
48h vs 144h	-0.087	-0.392 to 0.219

*Tukey error protection to control the chance of committing a Type I error (assuming significance in the absence of significance).

RESULTS

Table 1 summarizes the descriptive statistics for each group of serum samples (time periods between blood sample collection and centrifugation of 1h, 24h, 48h and 144h). Box-plots were used for comparative assessment of the data (Fig. 1). Figure 1 and the data from Table 1 clearly show an increase between the means and medians of CDT from the 1h group to the group with a time lapse of 144h between blood sample collection and centrifugation. The CDT data in each group were then tested for normal distribution. Frequency histograms were computed (not shown), and normality was checked by the Shapiro-Wilk W test. Because the coefficients of Shapiro-Wilk W, skewness and kurtosis did not differ significantly from their ideal values (1 for Shapiro-Wilk W and zero for skewness and kurtosis), the CDT data were considered to be normally distributed within each group. Thus, classical analysis of variance (ANOVA) could be used for testing the significance of differences between the means of CDT levels in the 4 groups with different whole blood storage time (1 h to 144 h). The p-value obtained was 0.042 which indicates a statistically significant effect of the time between the collection of the blood sample and centrifugation on serum CDT (Table 2). The statistical evaluation was extended further by analysis of contrasts between the groups. This indicates which groups differ from which other groups and which time period is critical for obtaining elevated serum CDT results (Table 2), which proved to be 144 h. Using 1-way Kruskal-Wallis ANOVA as the non-parametric, conservative variant of ANOVA, the results of parametric ANOVA were confirmed (p-value 0.038).

DISCUSSION

Information on the preanalytical phase of CDT analysis (e.g. the effects of patient pre-treatment, blood sampling, blood processing, sample storage and transportation) is scarce [1]. As is generally the case, adequate preanalysis is a precondition for the correct quantification of the analyte and reliable interpretation of the results. Therefore, the preanalytical phase of CDT determination merits further investigation. The present study demonstrates the importance of this issue by demonstrating increasing CDT values with time between blood sample collection and centrifugation (preparation of the serum). At least 2 explanations for this CDT increase can be given: in vitro degradation of the transferrin carbohydrate moieties by enzymes, and thus formation of sialic acid-deficient transferrins with isoelectric points close or equal to CDT isoforms, or interference of hemolytic serum with CDT determination. Indeed, hemolysis increases with the time between blood sample collection and centrifugation. However, there are several arguments supporting the hypothesis that there is a real in vitro CDT-increase: CDT analysis by the ChronAlcoI.D. test (and by other commercial CDT tests) consists of serum sample dilution, followed by CDT- and non-CDT-fractionation with anion-exchanger microcolumns and subsequent turbidimetric immunoassay of CDT transferrins. Although there were distinctly hemolytic serum samples in this study (free hemoglobin up to 1365 mg/L), the final microcolumn eluates used for CDT quantification were always visually non-hemolytic (clear and colorless).

This independence of the assay from hemolysis is further supported by the experience from our routine laboratory work (100–150 CDT tests per day). For a subset of 72 serum samples, free hemoglobin concentration was analyzed and the correlation between free hemoglobin and serum CDT concentration was tested by Spearman rank correlation. In this subset, CDT ranged from 1.5% to 4.2% (mean 2.3%, median 2.2%) and free hemoglobin from 13 mg/L to 1365 mg/L (mean 160 mg/L, median 70 mg/L). The Spearman correlation coefficient was $r = 0.15$ (95% confidence interval from –0.09 to 0.37), the two-tailed p-value was 0.21, indicating that there is no correlation between free hemoglobin and serum CDT concentration.

An in vitro increase of serum CDT therefore seems to be the most likely explanation for the higher CDT results after prolonged whole blood storage. Highly sensitive and specific isoelectric focusing analysis, which is usually referred to as the reference method of CDT isoform analysis [3], might provide additional insight into the processes occurring during the storage of whole blood; however, this was beyond the scope of the present study. From ANOVA with analysis of contrasts it follows that the CDT increase becomes significant only after 144h between blood sample collection and centrifugation. However, the data from Table 1 and Fig. 1 point to a slight (though not statistically significant) increase in CDT even after shorter whole blood storage periods. Since statistics are attributable to the population tested but not to the single individual, even this slight increase might already have consequences for the person tested – a shift from normal to borderline or from borderline to pathological CDT concentration. Therefore, we have extended the borderline for serum CDT concentrations indicating chronic alcohol abuse from the former 2.5–2.7% [2] to 2.5–3.0% in our CDT reports. This may further diminish the diagnostic sensitivity of CDT as a marker of chronic alcohol abuse. However, because of the potentially serious social consequences of a pathological CDT result, specificity is in most cases prior important than sensitivity.

In any case, the laboratory diagnosis of chronic alcohol abuse should always be made based on clinical background, questionnaire, CDT and γ-GT activity (measured at two different occasions), and not on a single CDT value.

CONCLUSIONS

A time period of several days between blood sample collection and centrifugation causes a significant increase in serum CDT and bears the risk of shifting initially normal CDT values (<2.5%) to borderline (2.5–3.0%) and initially borderline to pathological (>3.0%) CDT results. The date of blood sample collection and the receipt of the blood tube in the laboratory should be given in the CDT report and taken into consideration when interpreting CDT results.

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